A Retrospective Study of Equine Liver Lesions in Western Canada and the Expression of Metallothionein by Immunohistochemistry

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ABSTRACT

Liver disease in equids is often difficult to diagnose, as clinical symptoms are vague and do not appear until there is a functional loss of approximately 75% of the liver. Metallothionein (MT) is a ubiquitously expressed protein with high affinity for binding zinc in the liver. It has been implicated in inflammatory and neoplastic processes in various species, but its role has not been evaluated in horses.

To properly appreciate the possible implications of MT expression within the liver, it is crucial to understand, globally, the types of hepatic lesions to which equids are susceptible. Therefore, the first half of this work aimed to describe the histopathologic lesions of the liver from equids of all life stages submitted to the Prairie Diagnostic Services Inc. diagnostic laboratory (Saskatoon, SK) from 1995 to 2014, inclusive. Statistical analysis showed that the odds of being diagnosed with suppurative to mixed hepatitis was greater in juveniles and yearlings (odds ratio (OR) for both = 2.9, 95% confidence interval (CI) = 1.45 to 5.62 and 1.17 to 7.00, respectively), compared to adults. The odds of being diagnosed with multi-focal random hepatocellular necrosis were greater in fetuses and juveniles than in adults (OR = 77.1 and 6.1, CI = 22.6 to 263.2 and 2.1 to 17.8, respectively). The odds of being diagnosed with portal fibrosis and bile duct proliferation was greater in adults than in juveniles (OR = 5.4, 95% CI = 1.2 to 23.6). Neoplasia was diagnosed only in adults, and this was statistically significant (chi-square, P = 0.042). Additionally, equids such as donkeys, ponies, Miniature Horses as well as Coldbloods had significantly increased odds for the presence of hepatocellular vacuolation (OR = 29.5, 15.7, 7.4 and 7.9, 95% CI = 5.3 to 164.2, 2.7 to 90.8, 1.3 to 4.0, and 1.06 to 58.6 respectively). The odds of being diagnosed with suppurative to mixed hepatitis increased by each calendar year (OR = 1.07, 95% CI = 1.02 to 1.13).

The second half of this work focused on investigating the expression of MT in the equine liver as detected by immunohistochemistry. In particular, the relationship between MT expression and histopathologic features of liver disease was examined. These histopathologic features included hepatic inflammation, fibrosis, and bile duct proliferation. Also, the relationship between MT

expression and cellular regeneration, as determined by Ki-67 immunoreactivity, was determined. Metallothionein expression within hepatocytes was increased in 73 of 77 (94.8%) cases with chronic equine liver lesions. Statistical analysis found that MT expression was significantly associated with Ki-67 expression within bile duct epithelium (P = 0.0004, Mann-Whitney U-test), resident Kupffer cells (P = 0.0045, Mann-Whitney U-test), as well as with the presence of lymphocytic foci (P = 0.0017, Mann-Whitney U-test), suggesting that MT may play a role in inflammation and the cellular regeneration of bile duct epithelium in horses.

In conclusion, MT is associated with inflammation and cellular regeneration in equine liver disease. Additionally, the hepatic lesions described herein from animals from western Canada, though regionally specific, are in alignment with what we know about liver disease in equids. From these findings, and from what we currently understand about the pathophysiology of equine liver disease, there is an excellent opportunity to pursue further research into the development of MT-based diagnostics, preventatives, and therapeutics.

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DEDICATION

This thesis is dedicated to all the women in science: past, present, and future. May you forever continue to dream big and go after what is rightfully yours.

"All adventures, especially into new territory, are scary."

-Sally Ride

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LIST OF ABBREVIATIONS

° C	degrees Celsius
CCl_4	carbon tetrachloride
CI	confidence interval
Da	Dalton
DAB	diaminobenzidine
DIC	disseminated intravascular coagulopathy
ECM	extracellular matrix
EHV-1	equid herpes virus-1
FAT	fluorescent antibody test
H&E	hematoxylin and eosin
HPC	hepatic progenitor cell
IHC	immunohistochemistry
IFNγ	interferon-gamma
IL-6	interleukin-6
IQR	interquartile range
LPS	lipopolysaccharide
MEED	equine multisystemic eosinophilic epitheliotrophic disease
MMP	matrix metalloprotease
MRE	metal response element
MT	metallothionein
MTF-1	metal regulatory transcription factor-1
µm	micrometer
OR	odds ratio
PAS	periodic acid Schiff's
PDS	Prairie Diagnostic Service, Inc.
PHx	partial hepatectomy
qRT-PCR	quantitative real-time polymerase chain reaction
SCC	squamous cell carcinoma

sd	standard deviation
TNF-α	tumor necrosis factor-alpha
WCVM	Western College of Veterinary Medicine
WT	wild-type

CHAPTER 1: GENERAL INTRODUCTION

Hepatic disease occurs in a multitude of animal species, including humans. It can have significant implications for the animal, and mortality from liver disease is a real threat. This likely stems from the fact that the liver has important physiological roles that are crucial to the overall functioning of the organism. In addition, its unique vascularization from both the systemic and portal circulation exposes the liver not only to systemically circulating antigens, but also to gastrointestinally-derived micro-organisms, their products, and inflammatory mediators. The liver forms the final defense against these disease-causing agents before they enter the systemic circulation. The liver is therefore vital as an immune organ and its contribution to the inflammatory process results in the production of numerous pro-inflammatory mediators. These mediators, however, are also harmful to the cells of the liver itself and are the cause of additional damage during disease.

There is very little information about the occurrence of equine liver lesions in western Canada. Differences in breed preference, management practices, grazing areas and food supply may result in regional differences in the occurrence and distribution of disease entities. Patients with severe hepatic fibrosis often have a poorer prognosis as the liver is unable to regenerate adequately. Treatment of liver disease in equids is primarily supportive, to aid the liver in regeneration.

Metallothionein (MT) has been a hot topic of research since its discovery in 1957. It is a metallo-protein and expressed widely in a variety of species. Metallothionein is expressed constitutively in many organs and tissue types, especially the liver. Research has focused predominantly on its potential as an anti-inflammatory agent, but it has also been shown to be involved in hepatocellular regeneration, as well as the regression and resolution of hepatic fibrosis. It has been implicated in numerous human tumors, both as a negative and positive prognostic indicator, and its expression has been evaluated in small animal mammary and melanotic tumors. Current research is focusing on MT as a prognostic marker, especially in human neoplasia, as well as its value as a therapeutic agent, for example as a gene therapy.

The following thesis aimed to evaluate the role of MT within the current knowledge of equine liver disease. Chapter 2 provides a comprehensive literature review, discussing what we currently understand about liver disease in general, the potential role of MT within liver disease and liver disease in equids. Chapter 3 briefly summarizes the rationale, objectives, and hypotheses of this thesis. The main chapters of this thesis (Chapters 4 and 6), were written as manuscripts to be submitted for publication, with a short introduction preceding Chapter 6. Chapter 4 contains the results of a 20-year retrospective study of submissions containing hepatic lesions submitted to Prairie Diagnostic Services, Inc., and includes a comparison of those findings to what is currently known about equine liver disease. Chapter 6 focuses on the role of MT in chronic hepatic lesions in horses, and its correlation with histopathologic features of liver disease, such as inflammation, bile duct proliferation, and fibrosis. Additionally, the relationship between MT expression and cellular regeneration within the liver was explored. The final chapter contains the summary and conclusions of these studies, in addition to some thoughts on possible future endeavors in this exciting field.

CHAPTER 2: LITERATURE REVIEW

2.1 The Basic Structure and Functions of the Liver and Its Role in Disease

The liver arises early in embryogenesis from an out-pouching of endoderm that later forms the duodenum.[60] By adulthood, it is the largest solid organ in the body and weighs approximately 1.5 kg in humans and represents 2% of adult body weight.[24] In horses and other herbivores, the liver constitutes roughly 1% of body weight,[60] or about 5 kg in an average 500 kg equid.

Histologically, the liver has classically been described as being organized into lobules in which hepatocytes (hepatic epithelial cells) are arranged in cords around a central vein (also referred to as the terminal hepatic venule). Venous sinusoids are located between hepatic cords. Along the periphery of the lobule are portal triads consisting of bile ducts, branches of the hepatic artery and portal vein, nerves and lymphatic vessels, within a network of collagenous connective tissue.[60] The liver has a dual blood supply, receiving 75% to 80% from the gastrointestinal tract (portal circulation) and 20% to 25% arterial blood through the hepatic arteries.[24] The blood exits the liver via the sinusoids and central and hepatic veins to the vena cava.[24] The organ is surrounded by a fibrous capsule also known as the Capsule of Glisson.

Hepatocytes (hepatic parenchyma) are the most numerous cell type within the liver and occupy 78% to 80% of the total liver volume.[23] Other cell types associated with the parenchyma include intra-sinusoidal Kupffer cells, pit cells, sinusoidal endothelial cells and hepatic stellate cells within the Space of Disse.[309] The sinusoids, which carry portal blood toward the central vein, are lined by unique fenestrated endothelial cells arranged in sieve plates and lack a basal lamina.[256,308] The fenestrae act as a selective sieve, allowing only molecules of certain sizes through, and inhibiting the passage of chylomicrons greater than 200 nm to 250 nm.[256] Species-specific differences in the number and diameter of fenestrae have been demonstrated in several animals,[256] though no data could be found for equids. Additionally, endothelial cells have high endocytic activity, acting as a "scavenger" mechanism for waste molecules from the blood.[256] Hepatic stellate cells are mesenchymal cells that can transform from quiescent lipid and vitamin-

A storing cells to active myofibroblasts through extracellular signals from inflammatory cells, including Kupffer cells.[283] Pit cells are found within the hepatic sinusoid and are frequently found adhered to endothelial cells. These cells have cytotoxic activity and act as liver-specific natural killer cells.[174] Kupffer cells, which are resident macrophages within the hepatic sinusoids and occasionally within the Space of Disse,[256] have high phagocytic activity and can modulate the immune system through excretion of bioactive factors, including reactive oxygen species, inflammatory and growth control mediators.[234] The location of Kupffer cells within the sinusoid makes them the first cells of the innate immune system to come in contact with gastrointestinal-derived foreign and noxious material such as infectious agents, their products (including lipopolysaccharide, LPS) and other toxic substances.[256]

In general, the liver serves the following physiologic functions:

The liver plays a central role in carbohydrate and lipid metabolism. It removes ingested carbohydrates and triglycerides that arrive from the gastrointestinal tract via the portal circulation. Energy is stored in the liver as glycogen or exported to other organs as fatty acids.[60] Glycogen makes up approximately 5% of the wet weight of the liver,[293] which would correspond to about 250 g in a 500 kg equid. Energy is generated in the form of adenosine triphosphate through glycolysis, and fatty acid oxidation and approximately 28% of the total hepatocyte volume is taken up by mitochondria.[23] The liver is also the primary site of cholesterol synthesis, and degradation.[60] Cholesterol synthesis is dependent on the amount of dietary cholesterol intake. However, there is variability in the rate of cholesterol synthesis, as well the hepatic contribution thereof, between different species.[75]

Synthesis of several plasma proteins also occurs within the liver. These proteins include albumin, ceruloplasmin, lipoproteins, proteins of the coagulation cascade (factors II, V, VII to XIII), some acute phase proteins and those of the complement system.[60] Nitrogen metabolism is also completed here, with the conversion of toxic ammonia to non-toxic urea which is excreted by the kidneys into the urine.[60]

The liver has an important immune function. For example, hepatocytes produce acute phase proteins and coagulation factors which are involved in the systemic inflammatory response.[60] Additionally, Kupffer cells and natural killer cells are crucial components of the innate immune system and are first in the line of defense against foreign and noxious materials in the liver. Kupffer cells are in a prime position to phagocytize pathogens and foreign materials from both portal and arterial circulation and, therefore, form a major defense against gastrointestinally-derived immunogenic materials.[78] Phagocytosis of foreign material, including bacterial endotoxin,[99] results in the release of numerous bioactive compounds involved in the inflammatory response.[71,172] These include interleukins, interferons, arachidonic acid metabolites, leukotrienes, nitric oxide, among others.[71,172] Natural killer cells make up approximately 20% to 30% of the liver's resident lymphocytes.[79]

One of the main functions of the liver is the excretion of bile. Bile is an aqueous solution containing cholesterol, bile salts, bilirubin, amino acids, steroids, and enzymes as well as vitamins, heavy metals, and toxins.[29] Bile is produced by hepatocytes from cholesterol, excreted into bile canaliculi (formed by tight junction-sealed apical membranes of adjacent hepatocytes) and drains into bile ducts, making the liver an exocrine organ.[29] Bile is modified by the bile duct epithelium by way of an energy-dependent transport system that creates an osmotic gradient.[29] The principal functions of bile are the excretion of cholesterol and (harmful) lipophilic substances, the emulsification of dietary lipids and the absorption of fat-soluble vitamins (A, D, E, and K).[60] Bile also serves to transport immunoglobulin A to the mucosal surface of the gastrointestinal tract.[29]

The chemical modification of drugs, pollutants, and other potentially harmful molecules for removal from the body is an important task of the liver. Enzymes of the smooth endoplasmic reticulum within hepatocytes are responsible for the transformation of these molecules into metabolites that are more easily excreted, often by making them more water soluble.[5,233] The modification process starts with cytochrome p450 enzymes in a process known as phase I metabolism and is followed by phase II metabolism where conjugates are added through a different set of enzymes. Reabsorption of some metabolites from the gastrointestinal tract occurs

in a process known as enterohepatic circulation.[5]

The liver also serves as an endocrine organ, producing insulin-like growth factor-1 and angiotensinogen.[95] It is also involved in the metabolic transformation of sterol and thyroid hormones, for example, the conversion of thyroxine to triiodothyronine or androgens to estrogens.[9]

As with all other organs, the liver can be affected by primary disease. However, because of its involvement in numerous processes, as outlined above, and its unique exposure to both the portal and systemic circulation, it is also particularly susceptible to secondary disease. Hepatic involvement in gastrointestinal tract disease is not uncommon.[35] The inflammatory mediators that the liver produces are not only crucial in the clearing of bacteria and other immunogenic material but can also create hepatic inflammation and damage.[312] Despite the myriad of disease-causing agents and processes, the liver reacts in limited and predictable ways to injury. These include inflammation, fibrosis, biliary hyperplasia, and regeneration.[60] As such, it is often difficult to discern primary versus secondary hepatic disease on histology alone.

2.2 Reaction Patterns of the Liver in Disease

2.2.1 Hepatic inflammation

Human hepatic diseases in which inflammation is central to the disease process include: alcoholic liver disease, nonalcoholic steatohepatitis (progressive form of nonalcoholic fatty liver disease),[11] ischemia and reperfusion injury, parasitic infection, and viral hepatitis.[11,146]

Kupffer cells make up nearly 15% of all cells in the liver.[28] Kupffer cells are paramount in the initiation and progression of the inflammatory response, both locally within the liver and the systemic response. The myriad of pro-inflammatory, biologically active products produced by activated Kupffer cells is reviewed extensively by Decker.[71] The pro-inflammatory mediators produced by Kupffer cells may have deleterious effects on normal hepatic constituents and, therefore, are important in the development of hepatitis.[21] Blockage of Kupffer cell function by

gadolinium chloride resulted in a decrease in both hepatocellular necrosis and inflammation after (hepatotoxic) cadmium exposure.[77,241] Natural killer T cells play a co-stimulatory role, along with Kupffer cells, and induce the Type-1 T-helper (cell) response. Later, during chronic inflammation, there is a shift to cytokines that induce the Type-2 T-helper (cell).[128] Sinusoidal endothelial cells, hepatic stellate cells, natural killer cells, and hepatocytes also produce inflammatory mediators that coordinate with Kupffer cells to recruit neutrophils.[74]

The liver is involved in response to sepsis and critical for the clearance of bacteria and toxins from the bloodstream.[226] In one study, patients with sepsis had liver lesions that included portal inflammation, lobular inflammation, centrilobular necrosis, hepatocellular necrosis, cholangitis, and steatosis.[163] Hepatic dysfunction occurs early in sepsis and includes decreased glucose and xenobiotic metabolism, but hepatic lesions may not be apparent at this time. Later in the septic process, and as a response to decreased hepatic function, hepatocellular damage, cholestasis and suppurative inflammation occur.[201] During sepsis, the liver is responsible for several functions including production of proinflammatory cytokines, coagulation factors, complement and other acute phase proteins.[312] Neutrophils are attracted to the liver by the production of chemokines secreted by Kupffer cells, and together with platelets, Kupffer cells, sinusoidal endothelial cells, and hepatic stellate cells help clear bacteria.[310] Some B lymphocytes also display phagocytic activity.[232]

2.2.2 Hepatic fibrosis

In humans, hepatic fibrosis may develop in response to viral hepatitis, therapeutic agents, alcohol abuse, autoimmune disease, non-alcoholic steatohepatitis, or metabolic disease.[15] It is considered a form of wound healing, whereby the “scar” is characterized by the exuberant accumulation of extracellular matrix (ECM). There is a shift in the composition of the ECM during fibrosis, from basement membrane matrix to matrix containing dense fibrillar collagen.[100]

Hepatic fibrosis has traditionally been viewed as an irreversible process, even if the underlying cause is removed.[101] More recent evidence has demonstrated that fibrosis is reversible, at least in part, in some cases. For example, there was a significant reduction in hepatic fibrosis in the

majority of patients after alleviation of stenosis of the common bile duct.[120] Also, fibrosis can be reversed or stabilized in up to 80% of patients with chronic hepatic inflammation.[64] The use of anti-inflammatory drugs also highlights the importance of inflammation in the development of hepatic fibrosis. Reversal or stabilization of hepatic fibrosis is, however, not consistent in every case and the mechanisms of this are still being worked out.

Fibrosis occurs after chronic injury to the liver. Initially, acute hepatocellular damage results in regeneration of hepatic parenchymal cells. However, during chronic injury, regeneration fails and the hepatic parenchyma is substituted with abundant ECM.[15] Hepatic stellate cells are activated after injury and transform to myofibroblasts that are able to migrate to the area of injury and produce large quantities of ECM.[181,191] Kupffer cells are one of the major sources of transforming growth factor-ß1, which stimulates stellate cells to transform into myofibroblasts.[161] Myofibroblasts present within the portal area may also contribute to production of ECM.[156]

Reversal of hepatic fibrosis may occur after removal of the insulting agent and has been observed in experimental models and human patients.[64,136] Collagenolysis occurs by interstitial matrix metalloproteinases (MMPs), activated due to the decrease in the expression of tissue inhibitor of metalloproteinase-1.[136] Furthermore, the remodeling of the ECM may result in apoptosis of hepatic stellate cells.[136] Alternatively, reversion of hepatic stellate cell activation may occur by interleukin-10, which has been shown to down-regulate inflammation and increase collagenase activity.[279,296]

2.2.3 Hepatic regeneration

The liver is unique in its ability to regenerate. Unlike other organs, two-thirds of the liver can be removed without clinical consequences, and it returns to normal mass within the span of a few weeks.[124] Unfortunately, the liver is unable to regenerate fully after chronic injury, whereby the functional hepatic parenchyma is replaced by connective tissue.[25] After an injury, hepatocytes are the first cell type that enters the cell cycle and undergoes mitosis, followed by the proliferation of biliary epithelial cells, hepatic stellate cells, and Kupffer cells.[230] However,

hypertrophy of hepatocytes occurs before cell division in models after 70% partial hepatectomy (PHx). In models of 30% PHx, hepatocytes infrequently enter into mitosis, and organ mass is regained predominantly by hypertrophy.[196] Biliary hyperplasia (discussed below) through hepatic progenitor cells (HPCs), may form an additional line of regeneration. Hepatic progenitor cells (previously referred to as oval cells), with proper growth factors produced by activated hepatic stellate cells,[91] differentiate into basophilic hepatocytes within four to five days.[91,92] In one study, in patients with severe hepatic impairment, HPCs were activated after 50% loss of hepatocytes.[151] The HPC is also able to produce α-fetoprotein and albumin, suggesting an additional important physiologic function during hepatic dysfunction.[92] Angiogenesis is also initiated during hepatic regeneration to re-establish the hepatic vasculature.[25]

Coordination of hepatic regeneration involves numerous growth factors and other metabolic networks.[25,177] Tumor necrosis factor-α (TNF-α) binds to Kupffer and other non-parenchymal cells, which triggers the release of nuclear factor-κB, and interleukin-6 (IL-6).[97] Both can act on hepatocytes and are key in initiating hepatocellular regeneration through intracellular signaling pathways.[58,97] In turn, hepatocytes produce signals for other cell types to stimulate mitosis, including transforming growth factor-α, fibroblast growth factor-1 and -2, vascular endothelial growth factor, granulocyte macrophage colony-stimulating factor, and angiopoietin-1 and -2.[189,190]

Hepatic regeneration is impaired by numerous therapeutic agents, including chemotherapeutic drugs, statins, ß-blockers, non-steroidal anti-inflammatory drugs, and others.[162,243] In addition, after PHx in rodents, a period of hypoglycemia occurs, and supplementation of dextrose also impairs hepatic regeneration, suggesting that the regenerative response is coupled to the metabolic needs of the animal.[304] Regeneration ultimately leads to and stops, when there is 100% restoration of a species- and age-specific ratio of total body mass.[162,210,276]

Several animal models have been developed to study hepatic regeneration. Zebrafish are becoming more relevant models for developmental biology in general, and have been the focus of studies of liver development and disease research.[109,305] The most cited model is the rodent

model after PHx, in which the large and median lobes of the liver are surgically removed, resulting in loss of approximately two-thirds of the liver.[105,124,281] Rodent models involving chemically-mediated hepatotoxic injury include the use of carbon tetrachloride (CCL_4),[135] and D-galactosamine.[170] Hepatic injury caused by acetaminophen has also been studied in rodents.[158] Genetically modified animal models have also been developed in mice,[14] and swine.[122] The use of animal models has significantly furthered our understanding of hepatic regeneration in general.

2.2.4 Biliary hyperplasia

Bile duct proliferation, also referred to as bile duct hyperplasia or ductular reaction, is a reactive process that occurs with hepatic exposure to various insults, and is not limited to biliary injury. It involves proliferation of biliary epithelial cells and HPCs, and occasionally metaplasia of mature hepatocytes.[61] In animals, there are three recognized mechanisms for biliary hyperplasia.[61] Bile duct obstruction leads to a ductular multiplication of biliary epithelial cells within the portal stroma, and these small ducts contain no bile.[112] Leakage of bile into the surrounding tissue may result in recruitment of neutrophils. Chronic fibrosis and eventually cirrhosis will occur if the obstruction cannot be relieved.[112] In liver diseases in which there is severe damage to mature hepatocytes, and especially when they are unable to replicate, ductular reaction involves proliferation and differentiation of bi-potent HPCs.[61] These cells are located within the canals of Hering and are cytokeratin-7 and -9 positive. This corresponds to a biliary phenotype, despite a more prominent phenotypic hepatocyte morphology.[212] In contrast, mature hepatocytes are positive for cytokeratin-8 and -18.[93] With ductular reaction, progenitor cells replicate and differentiate to form numerous small and tortuous ducts. A final mechanism of ductular reaction described is under conditions of hypoxia when mature hepatocytes can undergo metaplasia and proliferation with an intermediate phenotype between cholangiocytes, and hepatocytes.[73] Ductular reactions may lead to parenchymal regeneration, fibrosis, and cirrhosis. They may also play a role in neoplasia, either through malignant transformation of the HPCs or by contributing to the development of a tumor microenvironment.[112]

In rats, bile duct proliferation with HPC proliferation is seen with D-galactosamine and CCl_4 induced hepatic damage.[170,253] and experimental fascioliasis.[173] Ductular reactions, both of the biliary replication and hepatocyte metaplasia variants, have been described in canine liver disease, including chronic hepatitis, cirrhosis, and cholangiocarcinoma.[131,314] In equines, biliary hyperplasia is seen with pyrrolizidine alkaloid toxicosis, aflatoxicosis, and serum hepatitis.[61] There are species-specific differences in the types of biliary reactions. Therefore, discernment is required when comparing animal and human disease models.[73,112]

2.3 Metallothionein

2.3.1 Introduction

Metallothionein (MT) was first isolated from the equine kidney in 1957 by Margoshes and Vallee as a cadmium-binding protein in the renal cortex.[180] It was later purified and further characterized by Kägi and Vallee in 1960.[147] Metallothionein is a ubiquitously expressed, low molecular weight (500-1400 Da), cysteine-rich, intracellular protein.[180] Because of its unique bioinorganic structure and ability to bind metals, it is referred to as a metalloprotein.[277] At the time of its discovery, MT's physical and structural characteristics were suggestive of a homeostatic biological role, such as catalysis, detoxification, or storage.[147] Early research focused on the role of MT in heavy metal detoxification, particularly that of cadmium.[297] Research into its potential role has since grown, and a recent PubMed search for "metallothionein" resulted in over 12,900 publications, with 207 publications in the first half of 2017. Research is focused on a wide variety of topics such as physical chemistry, molecular biology, and environmental pollution monitoring. Much of the applied medical research has involved studies on humans and research animals (predominantly rodents), involving both biochemical and genetic studies of its role. MT's true biological function, however, remains ill-defined, despite over 50 years of research.[214] It has been extensively reviewed in the literature.[57,69,119,133,134,198,214,239,277,278]

Metallothionein is a ubiquitously expressed protein and found in a large diversity of organisms including yeast,[299] fungi, and plants.[246] A review of MT in animals including mammals,

vertebrates, nematodes, annelids, mollusks, and arthropods, among others, is provided by Isani and Carpene.[134] In it, they describe the genetic heterogeneity between MT isoforms of different species, and that these differences do not appear to follow an evolutionary trend in that the least complex MT isoforms appear in more evolved vertebrates.[134] In primitive life forms, MT plays a role in the sequestration of toxic environmental metals.[57] For example, MT is constitutively expressed within the pharynx of *Caenorhabditis elegans* and may act to induce MT expression within intestinal cells upon heavy metal exposure.[125] The potential diversity of the function of the MT protein, based on its structure and biochemistry, is limited only by what is asked of it, from an evolutionary perspective. Therefore, the "true" function of MT is most likely dependant on the particular needs of that organism. It is frequently cited that there is difficulty in finding a unified role for MT, due to its specific and variable roles in different life-forms.[22,57]

There are four major groups of MT isoforms, which are classified based on numerous factors such as their molecular weight, the metal which binds, and genetics.[278] The major isoforms include MT-1 and MT-2, whereas the minor isoforms include MT-3 and MT-4. Metallothionein-1 and -2 are expressed during all stages of development and in most organs.[213] Metallothionein-3 was first isolated from brain neurons, and functions as a growth inhibitory factor.[288] Metallothionein-3 is also expressed in the male reproductive organs.[197] Metallothionein-4 was first isolated from differentiating stratified squamous epithelia.[229] It is also found in the upper gastrointestinal tract where it helps to regulate stomach pH, the sense of taste and texture on the tongue, as well as protects against UV damage of the skin.[278]

In humans, there are at least ten functional MT isoforms, of the four types, encoded by a family of 14 genes on chromosome 16.[302] In contrast, there are only four MT encoding genes on chromosome eight in mice.[229] This has implications for gene regulation, and that what we discover from mouse models may not apply to other species.

Transcriptional regulation of the MT gene is achieved in a variety of ways. Within the promotor are a number of regions responsive to different molecules. Metals, bound to transcription factors, bind to specific areas of the promotor known as metal response elements (MRE). For example,

activation by zinc of the MRE-binding transcription factor-1 (MTF-1) causes it to bind to DNA.[69] Free zinc can bind to the MRE via MTF-1, thus increasing MT mRNA.[295] Metallothionein DNA transcription is also regulated by stress, through glucocorticoid response elements within the MT gene promotor.[153] Hormones and cytokines may act on the promoter through intracellular signaling such as protein kinases.[69] For example, 1-alpha, 25-dihydroxy vitamin-D3 is known to increase the level of MT mRNA in mice when orally administered.[149] Bacterial endotoxin (LPS) is also known to increase MT mRNA, potentially through IL-6 and other cytokines in the mouse.[70] Additionally, DNA methylation of the promoter region is associated with suppression of MT gene expression in neoplastic hepatocytes in rats, and some other types of tumors.[106] Regulation of MT gene expression is achieved through many signals, both endogenous and exogenously produced.

Not only do MT isoforms show tissue specificity, but also specificity in the metals that they bind in those tissues. MT is bound predominantly to zinc in the human and equine liver.[34,227] However, it binds cadmium and zinc in nearly equal proportion in the human kidney.[227] The equine renal cortex has the highest levels of cadmium, in relation to other organs, and MT represents 1% to 2% of the total weight of soluble protein in the renal cortex.[147]

Metallothionein shows a wide variation in expression in different species and tissue types, and this concentration may be dependant on the age, diet, and developmental stage of the organism.[148] For example, human fetuses did not show evidence of MT expression by immunohistochemistry (IHC).[102] Metallothionein protein synthesis in rat intestinal mucosal cells is related to high levels of zinc in the diet.[185] In dogs, MT immunoreactivity has been demonstrated in glandular epithelia (mammary, uterine, sweat, olfactory, thyroid, and perianal), as well as fundic cells of the stomach, intestinal epithelial cells and hair follicle epithelial cells.[251] In equids, MT has been isolated and sequenced from the liver, kidney and intestine.[119,159,160]

Within the cell, MT is predominantly found in the cytoplasm. However, degradation of hepatic MT occurs predominantly within lysosomes, and the release of its bound metal is a prerequisite

for this process.[50] It has been observed that MT accumulates bound to copper within hepatic lysosomes of Bedlington Terriers with inherited copper toxicosis.[143] Transient translocation of MT into the nucleus has been seen in cells during proliferation and differentiation, and the elevated levels of MT in the nucleus may reflect an increase in demand for zinc by metalloenzymes and transcription factors, or to protect from DNA damage which may lead to apoptosis.[47,51,228]

2.3.2 Metallothionein and its role in inflammation

Metallothionein plays a very important role in inflammation and is considered to have anti-inflammatory properties. Human patients with diseases such as autoimmune or inflammatory bowel disease, cholestasis, and lymphoma had markedly increased expression of hepatic MT by IHC.[6] Bacterial endotoxin (LPS) can rapidly induce the transcription of hepatic MT in mice.[70] In addition, IL-6, TNF-α, and interferon-γ (IFN-γ) were also able to induce hepatic MT transcription, when injected intraperitoneally.[70]

The relationship of MT with the acute phase response has been investigated, and MT has been considered an acute phase protein because of its rapid response to inflammatory stimuli and association with other acute phase proteins. A significant relationship between MT and fibrinogen levels was found in mice injected with paraquat, menadione, and CCl_4 subcutaneously. Furthermore, this hepatic MT was bound to zinc.[194] The relationship between zinc levels and acute inflammation is well established. Zinc plays an important role in both the innate and adaptive immune system.[26,247] Hypozincemia has been seen during episodes of acute stress, such as during sepsis in which patients also have increased markers of oxidative stress and inflammatory biomarkers.[188] However, hypozincemia is associated with concomitant increases in hepatic zinc levels.[269] Hepatic levels of zinc increased in mice following LPS exposure, [259,269] but this accumulation did not occur in MT knock-out mice.[224] In addition, it is known that MT can sequester zinc in the liver during the acute stage of infection.[260]

The liver is a major contributor of inflammatory cytokines during systemic inflammation. The liver itself is susceptible to cytokine-induced pro-inflammatory injury, and this has been

investigated in a mouse model of thermal skin injury.[18,45,267] Metallothionein-I and MT-II mRNA and intranuclear protein levels were elevated after burn injury when compared to MT-knockout mice. Concurrently, increased levels of zinc, copper, and iron were also noted.[49] This suggests a role of MT in the pathogenesis of hepatic damage during the systemic inflammatory response. With regard to other organs, MT knockout mice were more susceptible to lung inflammation than wild-type (WT) mice when challenged with intratracheal LPS. Histologically, MT knockout mice showed degeneration of type I pneumocytes and endothelial cells, whereas the WT mice did not.[271]

2.3.3 Metallothionein and its role in fibrosis and biliary hyperplasia

The most recent research on MT has focused on the use of MT gene therapy. Hepatic fibrosis induced by CCl_4 was reversed in mice with the adenoviral delivery of the MT-2 gene.[140] Increased levels of MT were associated with increased collagenase activity, and there was increased hepatocyte regeneration after gene therapy.[140] Furthermore, MT-2 gene therapy was able to reverse the phenotype of activated hepatic stellate cells *in vitro*, thereby reducing the mRNA and protein levels of smooth muscle actin and collagen-1 from these cells.[311] In dogs with chronic hepatitis, MT expression was negatively correlated with the amount of fibrosis.[262] However, no such correlation was found in equids with chronic hepatic disease (refer to Chapter 4), suggesting a very species-specific mechanism for the potential anti-fibrotic activity of MT.

Matrix metalloproteases are important in the degradation of the ECM and for the reversal of hepatic fibrosis, as described earlier. Matrix metalloproteases are a family of endopeptidases and are dependent on zinc for their activity.[82,113,154] Is it plausible that MT plays a role in fibrinolysis and resolution of hepatic fibrosis? Indeed, treatment with luteolin in a CCl_4 mouse model of hepatic fibrosis resulted in the concomitant increase of MT-1 and MT-2 and MMP-9.[81] Furthermore, supplementation with zinc reduced liver fibrosis in mice as levels of MMP-13 and collagenase activity increased.[250]

There is no established relationship within the liver between MT and biliary hyperplasia. In one study by Schmitz *et al.*, MT overexpression was associated with poor patient outcome in cases of

cholangiosarcoma.[245] Metallothionein within bile duct epithelium in normal livers was occasionally only weakly expressed.[245]

2.3.4 Metallothionein in hepatic regeneration and neoplasia

Metallothionein expression has been positively correlated with hepatic regeneration (growth fraction) in dogs with chronic hepatitis.[262] In fetal and newborn rats, MT-1 and MT-2 are found in both the nucleus and the cytoplasm, which suggests a role for MT during phases of rapid growth.[43] Intranuclear localization of MT has also been observed in several human tumors, with more intense IHC staining of MT at the proliferating edge of malignant tumors.[46] In PHx rats, MT is highly expressed within the nucleus and is translocated from the cytoplasm rapidly after hepatectomy.[281,285] In addition, intra-nuclear staining of MT in hepatocytes was also shown within the S and G2/M phases of the cell cycle but intra-cytoplasmic staining occurred most prominently within the cytoplasm during G_0 and G_1 phases.[202,284,285]

In mice after PHx, the MT-1 and MT-2 knockout mice have significantly less hepatic proliferation than WT mice, suggesting an important role for MT in hepatic regeneration.[208] It is believed that MT functions as a storage pool and chaperone molecule for zinc, which is required by proliferating cells for transcription factors, growth factors, and metalloenzymes.[38,289,315]

2.3.5 Metallothionein expression as prognostic tool and the therapeutic potential of metallothionein

Metallothionein has been investigated as a potential biomarker in various neoplasms. Metallothionein expression has been evaluated in a variety of cancers such as breast cancer,[16,20,141,244] ovarian cancer,[268] renal cell carcinoma,[195] acute lymphoblastic leukemia,[242] lung carcinomas,[88] colorectal cancer,[205] pancreatic carcinoma,[206] and melanoma,[111,238,266,300] among others. In many instances, overexpression of MT is considered of prognostic value, as higher levels of MT within tumor cells was associated with increased risk for progression, and reduced patient survival in melanoma and cholangiosarcoma patients.[245,300] However, a recent meta-analysis of MT as an IHC biomarker showed a significantly elevated MT expression in (human) tumors of the head and neck (tissues of the oral cavity and tongue, pharynx, and larynx)

as well as in ovarian tumors, but a decreased MT expression in hepatocellular tumors. Furthermore, a significant positive relationship was found between MT expression and tumor grade and patient survival.[6] Within veterinary research, MT has been investigated in both mammary and melanoma neoplasms of dogs and cats. Although no prognostic implication was found in this study, it highlighted the species specificity in MT expression within neoplastic lesions.[76]

Interestingly, carcinogenesis and metastasis are also often correlated with an excess of metals such as copper and iron.[98] Tumor growth, angiogenesis and metastasis have been correlated to excess copper.[30,115] In turn, copper chelation was shown to inhibit copper-induced migration of neoplastic cells in prostate cancer.[217] Zinc chelation has been demonstrated to disrupt the conformation of the tumor suppressor protein p53 and was thereby able to modulate transcriptional activity.[187] Metallothionein, which binds heavy metals in the liver may, therefore, act as a source of cancer-promoting copper or zinc.

Although hepatic fibrosis is considered a reversible process, current therapeutic regimens against hepatic fibrosis have not provided a complete and consistent response, and therefore this is currently a "hot topic" of liver disease research.[64,301] The most recent research on MT has focused on identifying molecular targets,[261] as well as the use of MT gene therapy, [140,311] and other potential therapeutics, [80,81] for the reversal of hepatic fibrosis.

Of course, one must be judicious in interpreting results from mouse and *in vitro* models and applying them to human subjects. Although the liver has a limited number of reaction patterns, for example fibrosis, the etiologies and pathways to obtain that reaction pattern may be numerous and diverse. If one considers the number and type of immune cells and their inflammatory mediators, it would be foolish to consider all types of liver disease to be the same. By manipulating the expression levels of MT in the tissues of patients, we may be exposing them to increased risk of neoplasia.

2.4 Equine Liver Disease

2.4.1 Hepatic disease in the adult equid

In general, primary hepatic disease in adult equids is not common but may result in mortality in up to 25% cases.[87] In one study evaluating the cause of death of 241 mature horses, hepatic disease was only found in six horses.[192] Because of the liver's large reserve capacity, signs of hepatic disease may not become apparent until there is a loss of greater than 75% of liver tissue.[275] Very little information is available on the prevalence of hepatic disease in horses, except a few reviews out of the western United States,[116,192,216] and the United Kingdom.[83,86,184,303] Most of these reviews focus on the clinicopathologic correlation of hepatic disease with clinical signs, histopathology, or survival times, but do not relate findings to possible etiologies.

Hepatic disease in equids may result in transient or subclinical liver dysfunction or may cause more severe illness or death. Disease may follow an acute or chronic time course or may progress as a combination of the two, whereby an acute-on-chronic process may occur.

Primary bacterial hepatitis is rare in mature equids and is most often secondary to gastrointestinal disease. The bacteria are thought to ascend the bile ducts and gain access to the liver, resulting in a suppurative cholangiohepatitis.[222] Horses with proximal enteritis had significantly increased serum hepatic enzyme activity, suggestive of hepatic damage.[67,290] A recent case of necrotizing hepatitis, or black disease, caused by *Clostridium novyi* was described in a 20-year-old pony in western Canada.[66]

There are several other important causes of hepatic disease in adult equids. This includes lipidosis, especially in ponies, donkeys, and Miniature Horses.[104,130,139,199,200] Cholelithiasis with suppurative cholangiohepatitis has also been described.[144] Serum sickness, also known as Theiler's disease, is most likely caused by a Flavivirus, known as Theiler's Disease Associated virus.[44] Hepatic lesions involve acute hepatic necrosis and hepatitis, and the loss of hepatocytes may be so severe that the liver appears markedly reduced in size.[265] Infrequent causes of hepatic

disease include abscessation and iron toxicity.[209]

The nature of the grazing animal puts equids at risk for pasture and feed contaminated toxicities. Liver damage may result from the ingestion of pyrrolizidine alkaloid toxin containing plants,[3,186] alsike clover,[203] and aflatoxins.[36,63,292]

Granulomatous and eosinophilic lesions in the liver are most often a result of parasitic larval migration of *Strongylus vulgaris, Strongylus equinus, Strongylus edentatus, Parascaris equorum and Habronema sp.*.[183,263,286] Multi-systemic eosinophilic disease (MEED) has infrequently been reported in the literature, but may be a cause of eosinophilic granulomatous lesions in the equine liver.[41]

2.4.2 Hepatic disease in the juvenile equid

Clostridium piliforme is an important cause of hepatitis and death in foals less than 30 days of age.[270] The bacterium was first described by E. E. Tyzzer in a colony of waltzing mice in 1917.[287] Pathogenesis of the disease is due to the ingestion of the bacterium in fecal matter from adult horses, and its subsequent overgrowth in the nutrient rich intestine of foals.[270]

Toxic hepatopathy, described as massive necrosis and lobular collapse with mild portal fibrosis, has also been described in neonatal foals after administration of a nutritional paste containing *Aspergillus sp.* and an iron compound.[2]

Genetic diseases that can cause hepatic lesions are rare. However, a retrospective study from Berne, Switzerland, identified 30 Swiss Freiberger foals with hepatic disease consistent with congenital hepatic fibrosis, which is a genetically inherited disease in other species.[117]

Septicemia may cause secondary lesions of multi-focal hepatocellular necrosis in the liver. Foal septicemia is an important cause of illness in young animals and mortality may reach 75%.[164] *E. coli* is frequently cultured (blood culture) in septicemic foal cases.[164] Infection was the leading cause of morbidity in the first year of life for Thoroughbreds in Ireland, and septicemia was

diagnosed in 5.9% of foals, all occurring between 1 and 35 days of age.[103] Unfortunately, there are no specific symptoms for septicemia, and clinical signs of liver disease often do not accompany the clinical disease.[164]

2.4.3 Hepatic disease in the equine fetus

Few etiologic agents or disease processes cause significant hepatic pathology in the fetal liver. Equid herpes virus-1, and less frequently -4 (EHV-1 and -4), are causes of fetal abortion, often despite vaccination of the mare.[108,127,178,179,306] Equid herpes virus-1 was the leading cause in 21.3% of 103 cases of reproductive loss (abortion, stillbirth and early neonatal loss) in a study from central Italy,[178] 8.9% of 290 cases from Michigan,[273] and 6.5% of 1252 cases in a study from the United Kingdom,[108] and 3.3% of 1211 cases from a study out of Kentucky, USA.[127] Occasionally, there is co-infection of EHV-1 with significant bacterial isolates such as *Klebsiella pneumoniae* (lungs), or *Actinobacillus equuli* (septicemia).[179]

Hepatic lesions may be seen histologically in other disease processes. Equine viral arteritis predominantly causes lesions in the mare, but when present in the fetus, lesions include perivascular lymphocytic infiltrates or severe vasculitis involving the liver, spleen, lung, brain, and allantochorion.[72,142] Infectious agents responsible for placentitis may gain access to the fetal fluids or organs, which can result in hepatic lesions such as hepatocellular degeneration and necrosis.[306] Leptospirosis, most frequently due to serovars kennewicki or bratislava, is an important cause of fetal loss and will often result in gross and microscopic lesions in the fetus.[90,108,108,306] Hepatic lesions in the fetus include portal lymphocytic and histiocytic infiltrates with giant cells within the hepatic parenchyma.[306]

2.4.4 Neoplastic disease in equids

Neoplastic disease of the liver is uncommon in equids. While aged equids are susceptible to neoplastic lesions, and neoplasia resulted in the death or euthanasia of 18.7% of 241 equids in one study, no hepatic neoplastic disease was found.[192] Primary liver tumors described in older horses include cholangiocarcinoma,[84,254] a mixed hepatocellular carcinoma and cholangiocarcinoma,[150] and a single case of hepatic biliary adenofibroma (a variant of hepatic

biliary cystadenoma).[240] However, tumors metastatic to the liver are more common than primary hepatic neoplasms.[59] Multicentric hemangiosarcoma,[96] metastatic renal carcinoma,[157] and multicentric lymphoma[85] have all been described in the equine liver. In a retrospective study of 92 horses in the western United States performed by Hackett *et al.*, five cases of hepatic neoplasia were identified and included two cases of lymphosarcoma, an undifferentiated neuroendocrine carcinoma, one metastatic thyroid carcinoma, and one leukemia with neoplastic lymphoid infiltrates within the hepatic sinusoids.[116]

Primary hepatic neoplasia is even rarer in young animals. In equids, hepatoblastoma is one of the more frequently occurring neoplasms in fetuses and foals and has been described several times in the literature.[17,39,110,171,225,294] Hepatoblastoma is also one of the more common pediatric tumors in humans and accounts for 91% of primary hepatic tumors in children less than five years of age.[65] Other tumors described in equids include mixed hamartoma[237] and mesenchymal hamartoma[32] in fetuses and hepatocellular carcinoma in foals and young equids.[17,138,236]

2.5 Copper and Zinc in the Equine Liver

Copper and zinc play vital roles in the structure and function of the mammalian organism. Copper is essential for the functioning of enzymes,[121] as a structural component of tissues, and as an antioxidant and oxidant.[211] Similarly, zinc is important in enzyme function, immune function,[247] and for DNA and protein synthesis.[176] In humans, over 10% of the proteome codes for zinc-containing enzymes.[7]

Equids appear to be relatively resistant to toxicities caused by excessive dietary copper,[257] unlike what is observed in dogs,[94,255] sheep,[123] or other ruminants such as goats.[55] However, musculoskeletal deformities in foals and young horses have been attributed to copper deficiencies or with elevated zinc concentrations,[31,37,40,89,165] though copper has not been implicated in the pathogenesis of osteochondrosis dissecans.[298]

The main source of zinc and copper is diet, but the absorption of copper may be hindered by elevated concentrations of zinc.[152] There is a positive correlation between dietary copper intake

and hepatic copper concentrations in horses.[219] There appears to be no relationship between plasma and hepatic copper concentrations or plasma levels and dietary intake in equids.[62] Therefore, determination of the hepatic copper concentration is a better representation of copper metabolism, than plasma concentrations.[220] In contrast, mean hepatic zinc concentrations did not differ among a group of horses fed diets with varying amounts of zinc.[62]

A recent study (2014) by Paßlack et al.,[218] determined the naturally occurring heavy metal concentrations of copper, zinc, and cadmium, among others, of 21 horses in Germany. They found that both copper and zinc were higher, and in almost equal proportions, in the liver and renal cortex compared to the renal medulla. Cadmium was the highest within the renal cortex, followed by renal medulla and liver. No gender differences were found in the amounts of copper and zinc in both the liver and kidney. Copper concentrations in the liver were highest in young horses less than one year of age. In general, however, there was considerable individual variation in heavy metal concentration among horses.

CHAPTER 3: RATIONALE AND HYPOTHESES

3.1 Rationale

Metallothionein (MT) is a ubiquitously expressed metallo-protein found in a wide array of species and tissue types. Its expression is induced by various hepatotoxic insults, stressors and disease states. It has been shown to play an important role in inflammation, cellular regeneration, hepatic fibrosis, and neoplasia. However, even though it has been studied extensively in human medicine and rodent models of disease, there are species-specific differences in MT expression in health and disease. Hepatic disease in animals and humans often has a poor patient outcome, as the liver serves a multitude of functions that are critical to the life of the organism as a whole. The liver's involvement in inflammation is a double-edged sword, as the numerous pro-inflammatory mediators it can generate also are responsible for much of the damage caused by disease. As such, the inciting cause of the liver damage is rarely determined. Therefore, the current study aims to elucidate the role of MT in chronic hepatic lesions in horses, independent of the etiology, as well as increase our general understanding of histopathologic lesions and their potential etiologies in the equine liver.

3.2 Hypothesis 1

Hepatic histopathologic lesion characteristics and distribution within the local study population share similarities to what is currently known about hepatic disease in equids.

3.2.1 Objective 1

The objective of the first study is to evaluate, by way of retrospective analysis, lesions of liver disease in equids submitted to Prairie Diagnostic Services, Inc., from 1995 to 2014, inclusive, and to evaluate if the lesions are significantly associated with characteristics such as breed, sex, and life stage (fetus, juvenile, yearling, adult) in addition to submission year, season and collection method (biopsy, portion, whole-animal necropsy), and to compare the results with what is currently known about equine liver disease from the literature.

3.3 Hypothesis 2

Metallothionein is involved in the defense mechanisms against chronic hepatic lesions in equids.

3.3.1 Objective 2

The objective of the second study is to evaluate the hepatic defense mechanism mediated by MT in horses affected by chronic hepatic lesions by examining the correlation between hepatic MT expression level and the degree of hepatic inflammation, fibrosis, bile duct proliferation and cellular regeneration.

CHAPTER 4:CHARACTERIZING HISTOPATHOLOGIC LESIONS FROM EQUINE LIVERS FROM WESTERN CANADA: A 20 YEAR RETROSPECTIVE STUDY

This chapter contains the complete text of a manuscript that will be submitted for publication to *Veterinary Pathology*.

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4.1 Abstract

Knowledge about hepatic disease in equids is limited to a few retrospective analyses from the United Kingdom and the United States focusing predominantly on the clinicopathologic correlates of liver disease. This retrospective study was undertaken to describe histopathologic lesions of the liver from equids of all life stages, including aborted fetuses, submitted to the Prairie Diagnostics Services, Inc. laboratory in Saskatoon, SK from 1995 to 2014, inclusive. Furthermore, this study examined whether horse characteristics such as breed, sex and life stage (fetus, juvenile, yearling, adult), in addition to submission year, season and collection method (biopsy, portion, or whole animal necropsy), were significant characteristics for lesion diagnosis. Two hundred fifty-one equids, including ten donkeys, with hepatic lesions, were identified and the hematoxylin and eosin stained slides were reviewed. The median age of equids was six years old, and sex was evenly distributed. Quarter Horses were the most represented breed in this study, followed by Thoroughbreds. Overall, the most commonly diagnosed hepatic lesions were: suppurative to mixed hepatitis (88/251, 35.1%), multi-focal hepatocellular necrosis (36/251, 14.3%), and portal fibrosis with bile duct proliferation (32/251, 12.8%). Life stage was consistently statistically associated with several lesion diagnoses (suppurative to mixed hepatitis, multi-focal hepatocellular necrosis, portal fibrosis and bile duct proliferation and hepatic neoplasia), whereas sex, season and collection method were not. Breed was significantly associated with only one lesion diagnosis, hepatocellular vacuolation. Furthermore, a significant year to year increase in the number of submissions receiving a diagnosis of suppurative to mixed hepatitis was found in the evaluation period.

Key Words: equine, liver, biopsy, hepatitis, abortion, neoplasia.

4.2 Introduction

Liver disease is observed in equids and affects all breeds, ages, and genders.[41] Signs of hepatic disease are often nonspecific, making diagnosis a challenge,[11] and diagnosis relies primarily on clinicopathologic information such as serum biochemistry and biopsy.[3] The value of biopsy as a diagnostic tool is heavily dependent on the distribution of the disease process, as equine liver biopsy is routinely performed from the 13th intercostal space on the right side.[19] An ultrasound-guided biopsy is an option in those facilities with the equipment, yet even in cases of hepatopathy, up to 67% did not show ultrasonographic evidence of hepatic abnormalities.[86] Survival time has been demonstrated to be negatively associated with a high degree of neutrophilic inflammation, irreversible cytopathology or other chronic changes in biopsy samples,[87] suggesting that prognosis is dependent on lesion severity.

Liver disease in horses has a number of causes and may involve transient impairment of liver function or may lead to more severe illness or death. Reports on mortality vary but can reach up to 80% in affected animals.[116] Some of the more serious causes of liver disease in equids include, but are not limited to, serum hepatitis,[235] *Clostridium piliforme* (Tyzzer's disease),[221] lipidosis,[130] and cholelithiasis[126]. As horses are grazers, they are also naturally susceptible to liver damage caused by ingesting toxic plants such as those containing pyrrolizidine alkaloid toxins,[275] alsike clover,[203] and feed contaminated with mycotoxins.[63] Because of this, there may be regional differences in the type and frequency of liver lesions to which equids are susceptible.

Reproductive loss is a common problem and occurs in approximately 10% to 15% of pregnant mares.[306] The majority of abortions arise as embryonal loss early in pregnancy, with one peak of fetal loss at around 40 days of gestation and a second peak occurring in the final months of gestation.[306] Many of the infectious and toxic agents that can cause abortion may also result in lesions within the fetal liver. Some agents, such as equid herpesvirus-1 (EHV-1), target the liver specifically. Fetal sepsis is most often caused by bacterial agents and results in hepatic lesions early in the septic process.[312] Therefore, histopathologic evaluation of the fetal liver is a vital step in diagnosing the cause of abortion.

Few retrospective analyses on equine liver disease exist, the majority of these focus on clinical signs, serum biochemistry, and other clinicopathologic correlates.[83,86,184,216,303] Of those, the largest study included a group of 116 horses with naturally occurring disease in the United Kingdom.[86] Recent work by Hackett *et. al.*,[116] reviewed histopathology of 92 cases of hepatic disease in adult horses in the western United States. However, their study focused primarily on histopathologic lesions in relation to patient outcome and clinicopathologic data but did not discuss potential etiologies of those lesions. In addition, there are no published studies available that have evaluated the type and frequency of hepatic lesions in fetuses and juvenile equids.

The objectives of this study were to describe and characterize the variety of histopathologic lesions in the equine liver from western Canada submitted to Prairie Diagnostic Services, Inc. (PDS), a diagnostic laboratory in Saskatoon, Canada. The second objective was to investigate if individual horse characteristics (life stage, breed, sex), temporal factors (season, year of submission) and collection method (biopsy, portion or whole animal necropsy) were significant characteristics for lesion diagnosis in this local population. The final objective was to discuss potential etiologies, concurrent pathology, and ancillary testing when available and where appropriate.

4.3 Materials and Methods

4.3.1 Case selection

The records from submissions received by PDS between January 1, 1995 and December 31, 2014, were identified by a computer-assisted search and evaluated for histologic evidence of hepatic disease (search terms: liver, hepatic, hepatitis, necrotizing, necrosis, inflammation, fibrosis, bile duct proliferation, hemorrhage, lipidosis, cirrhosis, inclusion body, neutrophil, lymphocyte, and plasma cell). Submissions originated predominantly from the Canadian provinces of Saskatchewan and Alberta, although submissions from Manitoba and British Columbia were also included. Only those cases with histologic descriptions of the liver lesions were assessed, from both horses and donkeys. Hepatic histopathologic lesions from the pathology report were confirmed by reviewing the available hematoxylin and eosin (H&E) stained liver sections (JNCV and ANA). Reports with similar lesions were first grouped; then any statistical or ancillary analysis was performed.

Breed, sex, the age of the animal and collection method (biopsy, portions or full-body necropsy) were extracted from the record. If the sex or breed were unknown, or not evident from the record, the animal was recorded as “unknown.” If age was not specified, it was left blank. Very few individuals represented some breeds. Therefore, breeds were grouped into similar breed groups. “Quarter Horse” included similar breeds such as Appaloosas, American Paints, and Pintos. Chuckwagon ponies were placed together with Thoroughbreds. Breeds were further grouped as “Hotbloods” (Thoroughbred, Arabian, and Morgan), “Warmbloods” (Quarter Horse, Belgian Warmblood, Foreign Warmblood), “Coldbloods” (Percherons and Clydesdales), “Ponies” (all animals designated as pony, Welsh Pony, or Shetland Pony), donkeys (donkey and Miniature Donkey). All cross- and mixed-breeds, two Standardbreds, one Saddle Horse, one Icelandic Horse and one Tennessee Walking Horse, were placed in the same category with the unknown breeds, to improve statistical power. This “unknown” group became the reference group for statistical analysis. Life stage was determined based on the indicated age of the animal, or estimated from information from the submitted history or necropsy report if age was not specified. An adult horse was any animal over the age of 24 months, a yearling was 10 to 24

months (inclusive), and a juvenile (foal) was from birth to 9 months. Gestational age was extracted from pathology reports when available or extrapolated and estimated according to *Kirkbride's Diagnosis of Abortion and Neonatal Loss*[306] using fetal weight, crown-rump length, and other fetal and placental characteristics found in the report. From the date of the pathology report, year and season of submission were determined. "Spring" was defined as the months of March, April, and May, "summer" as the months of June, July and August, "fall" was defined as September, October, and November, and "winter" was defined as December, January, and February.

4.3.2 Statistical analysis

All statistics were performed using Stata 14.2 (StataCorp, College Station, Texas, USA) (JNCV and SP). The mean and standard deviation was reported as a measure of central tendency if age or fetal age followed a normal distribution. For lesions where age did not follow a normal distribution, the median and interquartile range (IQR) were reported. Univariate analysis was carried out for each lesion and was conducted using chi-square analysis. Each lesion was evaluated to determine if the characteristics (breed, life stage, sex, year of lesion diagnosis and season) were significantly associated with the occurrence of that lesion in comparison to all other lesion groups. Multi-variate analysis was further performed if potential significant characteristics were identified by univariate analysis and were included in the initial model at a relaxed level of significance ($P \leq 0.2$). The final model building included a more restrictive level of significance ($P \leq 0.05$). The odds ratio was calculated to compare the differences between groups of a single characteristic variable. Evaluation of model fit was performed by evaluating the Pearson goodness of fit test and examining the residuals for leverage. The likelihood ratio test was performed to compare full and reduced models to test for significant predictors.

4.4 Results

Two hundred fifty-one submissions of horses and donkeys with histopathologic hepatic lesions were identified. The range of submissions per year was 7 to 20, with 1995 having the lowest number of submissions and 2005 the highest (Figure 5-1). The mean number of submissions per year was 12.6 (sd = 3.12), and the median was 12.5. The winter season had the lowest number of submissions (54/251, 21.5%) compared to spring (65/251, 25.9%), summer (64/251, 25.5%) and fall (68/251, 27.1%). Only 32 of 251 (12.8%) submissions were collected by biopsy, with the remainder as (organ) portion submissions (78/251, 31.2%) and full-animal necropsies (141/251, 56%).

The life stage breakdown of the 251 animals included 152 (60.6%) adults, 24 (9.6%) yearlings, 50 (20.0%) juveniles, and 25 (10.0%) fetuses. The age range was 1 month to 31 years with a median of 6 years and IQR of 1 to 13 years. Twenty-four animals were specified only as adults, and six animals were determined to be juveniles. Fetal age was estimated in 12 cases, with a range of 180 days to 330 days gestation and a mean of 257.5 days (sd = 54.6 days). Thirteen fetuses did not have a gestational age estimation. Overall, there were 114 (45.4%) males, 119 (47.4%) females and 18 (7.2%) were of unknown sex. The breakdown of sex by life-stage is given in Table 5-1.

The specified breeds of the 251 equids are given in Table 5-2. Quarter Horses and related breeds were the most numerous known breeds represented (96/251, 38.3%), followed by Thoroughbreds (27/251, 10.8%) and other Hotbloods (Arabian, Morgan; 15/251, 6.0%).

Table 5-3 lists the frequency and percent proportion by life-stage of hepatic lesions in this study. Overall, the most commonly diagnosed hepatic lesions were: suppurative to mixed hepatitis (88/251, 35.1%), multi-focal hepatocellular necrosis (36/251, 14.3%), and portal fibrosis with bile duct proliferation (32/251, 12.8%). The most common lesion in adults, yearlings, and juveniles was suppurative to mixed hepatitis (46/152, 30.3%; 13/24, 54.2% and 26/50, 49.1%, respectively). Adult horses were also frequently diagnosed with portal fibrosis and bile duct

proliferation (28/152, 18.4%) and hepatocellular vacuolation (20/251, 13.2%). Juveniles had frequent cases of multi-focal hepatocellular necrosis (10/50, 20.0%). The only lesions characterized in fetuses were multi-focal hepatocellular necrosis (19/25, 76.0%), suppurative to mixed hepatitis (3/25, 12.0%) and centrilobular hepatocellular necrosis (3/25, 12.0%)

Specific histological lesions and their associated demographic characteristics are described in further detail in the following sections, from most to least frequent.

4.4.1 Hepatitis, suppurative to mixed inflammation

A predominantly suppurative hepatitis, with or without lymphocytes, eosinophils or macrophages was observed in 88/251 (35.1%) cases and was the most frequently observed lesion in this study. The distribution of the lesions was either primarily portal (cholangiohepatitis; 56/88, 63.6%), multi-focal random (29/88, 33.0%), or described as focal abscesses (3/88, 3.4%). Fibrosis and bile duct proliferation were commonly observed in the portal distributed lesions (38/56, 67.9%), and ranged from being mild and localized to extensive and bridging. Bile stasis was also commonly seen. Mild hepatocellular lipidosis, portal edema, and hepatic necrosis were infrequently present. Hepatocellular necrosis with occasional foci of mineralization was commonly associated with multi-focal parenchymal hepatitis.

Submissions occurred 1 to 10 times per year, with a peak of 10 submissions in 2013. Figure 5-2 shows the overall increasing trend in submissions over the study years (compare to Figure 5-1 which represents submissions for all cases over the study period). The lesion distribution was 46/88 (52.3%) in adults, 26/88 (29.6%) in juveniles, 13/88 (14.8%) in yearlings and only 3/88 (3.4%) in fetuses. The age range for 77 known ages was 1 month to 30 years, with a median age of 3 years and IQR of 7 months to 10 years. Fetal age was known in one case, at approximately 330 days of gestation. Both submissions per year and life stage were significantly associated characteristics as determined by multi-variate analysis for the diagnosis of suppurative hepatitis. The odds ratio increased 1.07 times per year for this lesion (P = 0.005, 95% CI = 1.02 to 1.13). Furthermore, juveniles and yearlings both had 2.9 times increased odds of being diagnosed with this lesion in comparison to adults (P = 0.002 and 0.021, 95% CI = 1.45 to 5.62 and 1.17 to 7.00,

respectively). When the full model was tested against a more constrained model containing life stage only, the likelihood ratio test confirmed that the full model was better fitted ($P = 0.0046$). One large residual was observed in the full model. However, when the observation was removed, and the logistic model was run again, neither the odds ratios nor the P-value changed significantly. This observation was therefore included in the final model.

Fall had the largest number of submissions (28/88, 31.8%), followed by spring and summer (21/88 each, 23.9%) and winter (18/88, 20.5%; Table 5-4). More than half were received as necropsies (45/88, 51.1%), 28/88 (31.8%) as portions and 15/88 (17.1%) were biopsy submissions. However, neither season nor collection method were significantly associated with a diagnosis for this lesion.

Bacterial culture from the liver was performed in only 21 cases (23.9%; Table 5-5). Bacterial culture was largely unrewarding, with over half (11) reporting a negative result. Clinically significant isolates were found in acute cases of hepatitis, with *Streptococcus equi* subsp. *zooepidemicus, Actinobacillus equuli*, *Listeria sp*., *Salmonella sp*. being cultured. *E. coli* was cultured in the liver in both acute and chronic cases.

Three cases of hepatic abscesses were diagnosed. One abscess was diagnosed in a seven-month-old juvenile horse and had a positive culture for *Streptococcus equi* subsp. *zooepidemicus.* The remaining two cases were diagnosed in adult horses, 9 and 19 years of age. Both were female Quarter Horses. One of the abscesses was attributed to a foreign body peritonitis, no cause for the other abscess could be determined. No additional bacterial culture was performed in these two cases.

Two cases of chronic cholangiohepatitis in an 8-year-old female Thoroughbred and a 13-year-old male Quarter Horse were due to cholelithiasis, with choleliths up to 10 cm in diameter found in the bile duct at necropsy. A 14-day-old male Thoroughbred had hepatitis associated with a concurrent omphalophlebitis.

4.4.2 Multi-focal random hepatocellular necrosis

In 36/251 (14.3%) submissions, lesions were characterized by multi-focal random coagulative hepatocellular necrosis, that was occasionally accompanied by mild inflammatory infiltrates.

Submissions occurred between 0 to 4 times per year, with peaks in 1996 and 1999. The diagnosis was most frequently made in the spring (20/55.6%) followed by summer and winter (7 cases each, 19.4%) and fall with 2 cases (5.6%; Table 5-4). Twenty-five cases (71.4%) originated from necropsies, nine cases (25.7%) from portions submissions and one case (2.9%) from biopsy. However, none of these characteristics were significantly associated with lesion diagnosis.

This lesion occurred most frequently in fetuses (19/36, 52.8%) followed by juveniles (10/36, 27.8%) and adults (6/36, 16.7%). Only one yearling was diagnosed with this lesion. The age range of 14 known individuals was between 1 month to 11 years of age, with a median age of 1 month and an IQR of 1 month to 5 years. Nine fetuses were given estimated gestational ages, ranging from 180 days to 330 days, with a mean of 264.5 days (sd = 42.9). Life stage was a significantly associated characteristic for diagnosis with this lesion ($P < 0.0001$). Fetuses had 77.1 times greater odds ($P < 0.0001$, 95% CI = 22.6 to 263.2) and juveniles had 6.1 times greater odds ($P = 0.001$, 95% CI = 2.1 to 17.8) when compared to adults.

All nineteen submitted fetuses also had intra-nuclear eosinophilic inclusion bodies most consistent with EHV-1 infection. However, there was also a single case of a six-year-old male donkey also with similar (viral) inclusion bodies. No further testing was submitted for this case. Additional diagnostic testing was performed on 14 cases of suspected EHV-1 in fetuses. Eleven cases had tissues submitted for a fluorescent antibody test (FAT), ten of which were positive and three cases had liver sections submitted for immunohistochemistry (IHC) and were all positive for EHV-1.

Five out of 36 cases (13.9%), all originating from juveniles four weeks of age and younger, contained intra-cytoplasmic groups of rod-shaped bacteria visualized on H&E or facilitated by silver stain, consistent with *Clostridium piliforme* infection within hepatocytes at the necrotic

edge. The exact etiology of hepatic lesions in the remaining five juveniles was unknown but was thought to have originated from a variety of etiologies: One case had a positive liver culture for *E. coli*, however, lung, spleen, and kidney all cultured *Streptococcus equi* subsp. *zooepidemicus* (consistent with sepsis), one other portion case was highly suspicious of sepsis, as significant lung lesions with intralesional bacteria were described (no culture was performed), and a third case was diagnosed histologically with a severe peritonitis, pleuritis, and meningitis (consistent with sepsis), though no bacterial culture was performed. The fourth case was described as having lung abscesses that cultured positive for *Streptococcus equi* subsp. *zooepidemicus* and the final case was of unknown etiology.

4.4.3 Portal fibrosis and bile duct proliferation

Hepatic lesions of portal fibrosis and bile duct proliferation was diagnosed in 32/251 (12.8%) cases and were characterized by portal fibrosis that was often extensive and bridging with bile duct proliferation (ductular reaction). Lesions may have also had mild hepatocellular necrosis, hepatocellular vacuolation, or inflammation. Megalocytosis of hepatocytes was reported in a single case, and bacterial culture was unrewarding when performed.

The range of submissions was 0 to 4 times per year, with a peak of 4 cases in 2011. These lesions were most often diagnosed in the fall (11/32, 34.4%), followed by summer (9/32, 28.1%), spring and winter (6/32 each, 18.8%; Table 5-4), though this was not found to be statistically significant for lesion diagnosis. Five cases were diagnosed on biopsy submissions (15.6%), with the remainder as portions submissions (14/32, 43.8%) and whole-body necropsies (13/32, 40.6%).

In total, 2 juveniles, 2 yearlings, and 28 adults were evaluated with this lesion. The age range was 1 month to 28 years, with a mean age of 11.6 years (sd = 7.1; 30 individuals with known ages). Seventeen males (53.1%) and 15 females (46.9%) were diagnosed. Life stage was a significantly associated characteristic for this lesion and adults had 5.4 times greater odds than juveniles (P = 0.025, 95% CI = 1.2 to 23.6). Two cases originated from the same farm and were both submitted only days apart in September of 1997. The remaining submissions were likely unrelated.

4.4.4 Hepatocellular vacuolation

Submissions of hepatocellular vacuolation included all cases potentially containing lipid and glycogen, and where vacuolation was the dominant lesion in the liver (25 cases, 10.0 %). In 19 cases, the vacuoles were interpreted by the pathologist based on histology to contain lipid, one was determined to be due to glycogen, three were interpreted as vacuolar degeneration or hydropic change, and two cases were not interpreted. Only four cases of presumed lipid vacuolation were tested with Oil Red O, and all were positive for lipid. The single case of suspected glycogen accumulation stained positively for Periodic Acid Schiff's (PAS) stain and was cleared by PAS-diastase, consistent with intracellular glycogen. Microscopically, cases of hepatocellular vacuolation consisted of intra-cytoplasmic, optically clear vacuoles which ranged from micro-vesicles to macro-vesicles. Vacuoles were of variable sizes and ranged from being distinct, and round to less distinct and irregular. Nuclear placement was either central within the cell or eccentrically located. Few cases also contained mild portal lymphocytic infiltrates, mild fibrosis, mild hepatocellular necrosis and lipofuscin pigment. Bacterial culture of the liver was performed in five cases and returned insignificant results.

Submissions of hepatocellular vacuolation occurred 0 to 3 times per year, with peaks of 3 cases in 1996, 2002, and 2005. It was most frequently diagnosed in the summer months (7/25, 28.0%) with the remaining cases spread equally over spring, fall and winter (6/25, 24.0%; Table 5-4), though this distribution was not statistically significant. Approximately half of the cases (12/25, 48.0%) were diagnosed on necropsy, 8/25 (32.0%) on portion submissions and 5/25 (20.0%%) on biopsies.

Hepatocellular vacuolation was seen in adults (20/25, 80.0%), yearlings (2/25, 8.0%) and juveniles (3/26, 12.0%) but not in fetuses. The age range of equids with the lesion was 1 month to 31 years, with a mean age of 9.9 years (sd = 7.8). Nine animals (36.0%) were male, 15 (60.0%) were female, and 1 was of unknown sex. Two individuals were also diagnosed with pituitary adenomas on necropsy.

Breed was a significantly associated characteristic for hepatocellular vacuolation. Donkeys (including Miniature Donkeys) had 29.5 times greater odds (P = 0.0001, 95% CI = 5.3 to 164.2), ponies had 15.7 times greater odds (P = 0.002, 95% CI = 2.7 to 90.8), Coldbloods had a 7.9 times greater odds (P = 0.044, 95% CI = 1.06 to 58.6) and Miniature Horses had 7.4 times greater odds (P = 0.026, 95% CI = 1.3 to 4.0) when compared to the mixed breed/unknown group. Only Percherons were diagnosed with hepatocellular vacuolation of those breeds included in the Coldblood group.

4.4.5 Centrilobular hepatocellular necrosis

Centrilobular hepatocellular necrosis was characterized in 24/251 (9.6%) of submissions. Histologically, all cases contained at least some centrilobular hepatocellular necrosis. Submissions may also have contained fibrosis, particularly of the terminal hepatic venule or sinusoidal lining, mild inflammation, and variable amounts of centrilobular (hemorrhagic) congestion. Of the 24 cases, 13 were due to heart failure, 10 of which had concomitant cardiac lesions. One case was due to neonatal isoerythrolysis. The exact etiology of liver lesions in the remaining 10 cases was unknown. However, three were suspected to be due to acute toxin exposure, and three were thought to be caused by an infectious agent resulting in disseminated intravascular coagulopathy (DIC), thrombosis or hypoxia due to severe pneumonia. Four cases were of unknown cause, and the hepatic lesions were considered unrelated to the determined cause of death.

Submissions were observed 0 to 3 times per year, with a peak of 3 submissions in 1999 and 2006 and were most often diagnosed in the winter (7/24, 29.2%), followed by spring, summer, and fall (Table 5-4). Season, however, was not a statistically significant associated characteristic of this lesion. Cases were all found on post-mortem exam (portions and necropsies).

Lesions were seen in adults (13/24, 54.2%), juveniles (3/24, 20.8%), yearlings (5/24, 20.8%) and fetuses (3/24, 12.5%). The age range of 17 known individuals was from 1 month to 27 years, with a mean age of 7.6 years (sd = 7.4). One fetus was determined to be approximately 180 days in gestation.

4.4.6 Non-specific portal lymphocytic infiltrates

Fifteen submissions (6.0%) with non-specific portal lymphocytic infiltrates were identified. Submissions were seen between 0 and 2 times per year and evenly distributed across the seasons (4 cases each in summer, fall, and winter, 3 cases in spring; Table 5-4). All cases were diagnosed on post-mortem exam (5 from portions submissions, 12 from necropsies). Microscopically, lesions were consistent with mild portal lymphocytic infiltrates, occasionally with eosinophils and in the absence of hepatic parenchymal damage and suppurative inflammation.

Adult equids were diagnosed in 12 cases (80.0%), with one yearling and two juveniles. Ten cases were males (66.7%), four females (26.7%) and one unknown sex. The age range from 10 known individuals was from 4 months to 15 years, with a median age of 7 years and an IQR of 1 to 12 years.

4.4.7 Focal or multi-focal hepatitis, granulomatous

In 15/251 (6.0%) cases, granulomatous inflammation consisting of macrophages, often with multi-nucleated cells and admixed with eosinophils was observed either focally or multi-focally. Fibrosis was frequently present (11 cases) and was associated with the inflammation, either as an increase in fibrotic stromal tissue or as a capsule surrounding granulomas.

Submissions occurred 0 to 2 times per year, most frequently in the summer months (7/15, 46.7%), followed by fall, winter (3/15 each, 20.0%) and spring (2/15, 13.3%; Table 5-4), though season was not a significantly associated characteristic. Nine cases (60.0%) were diagnosed from necropsies, three from portions and three from biopsies.

Twelve adults (80.0%), two yearlings (13.3%) and one juvenile (6.7%) were diagnosed, seven males (46.7%), seven females and one of unknown sex. This lesion was not observed in fetuses. The age range of equids with this lesion was 3 months to 17 years, with a median age of 11 years and an IQR of 5 to 12 years (of 13 known individuals).

In 13 cases, the lesions were consistent with parasite migration, though only a single case had evidence of a parasite (*Parascarus equorum)* within mineralized granulomas. The exact etiology of the remaining two cases was not determined, but one submission was suggestive of *Mycobacterium avium* granulomas, and one was highly suspicious of a *Rhodococcus equi* infection, both based on histopathologic lesions in other organs, though liver culture was negative.

4.4.8 Neoplasia

Hepatic neoplasia was diagnosed in 12/251 cases (4.8%). Half of the cases (6/12) were diagnosed in the fall months, with three cases seen in the winter, two in the summer and one in the spring (Table 5-4). Three cases (25%) were diagnosed on biopsy, with the remaining diagnoses made on post-mortem exam. Only adults were affected, and this was significant by chi-square analysis ($P = 0.042$). The median age of affected horses was 16 years with an IQR of 6 to 25 years (11 known ages, one unknown).

Multicentric lymphoma was diagnosed in 8/12 (66.7%) cases of neoplasia. The primary process was always of gastrointestinal origin. Three appaloosas, three Quarter Horses, one Quarter Horse cross, and one American paint were affected, of which five were male, two were female, and one was of unknown sex. The age range for diagnosis was 4 to 27 years of age, with a mean of 13.6 years and a median of 13 years.

One case of cholangiocarcinoma was diagnosed in a 26-year-old female mixed-breed horse. Necropsy also revealed metastasis of the neoplasia to the lungs.

Individual submissions of other (metastatic) neoplasia were diagnosed in the liver. Squamous cell carcinoma (SCC) was observed in a 17-year-old female Thoroughbred. Hemangiosarcoma was diagnosed in a 16-year-old female Quarter Horse. The primary tumor could not be identified, though neoplastic cells were observed in the muscle, thyroid, lung, kidney and adrenal gland, in addition to the liver. A single submission of renal cell carcinoma was diagnosed in an adult female Quarter Horse of unknown age.

4.4.9 Infarction

Three submissions of hepatic infarction were diagnosed. Microscopically, the lesions consisted of a focal area of coagulative hepatic necrosis and hemorrhage, and one submission had a significant neutrophilic inflammatory infiltrate. Two animals were observed to have a liver lobe torsion at necropsy, a 4-year-old female Belgian Warmblood and the other a 24-year-old male Quarter Horse. The third animal was a nine-year-old male Appaloosa and was a portions submission with no additional information on the necropsy findings.

4.4.10 Congenital microvascular dysplasia (shunt)

A single submission of a congenital vascular anomaly was diagnosed in an eight-month-old, male Quarter Horse. Histologically, there was abundant, diffuse arteriolar proliferation (multiple, prominent and tortuous) with hepatocyte atrophy and mild portal fibrosis.

4.4.11 Multi-focal eosinophilic granulomas

A single submission of multi-systemic eosinophilic epitheliotropic disease (MEED) was diagnosed on post-mortem exam (necropsy). Histologically, the liver had multi-focal eosinophilic infiltrates, as did other organs, including the skin. The affected animal was a four-year-old, female Quarter Horse.

4.4.12 Results figures

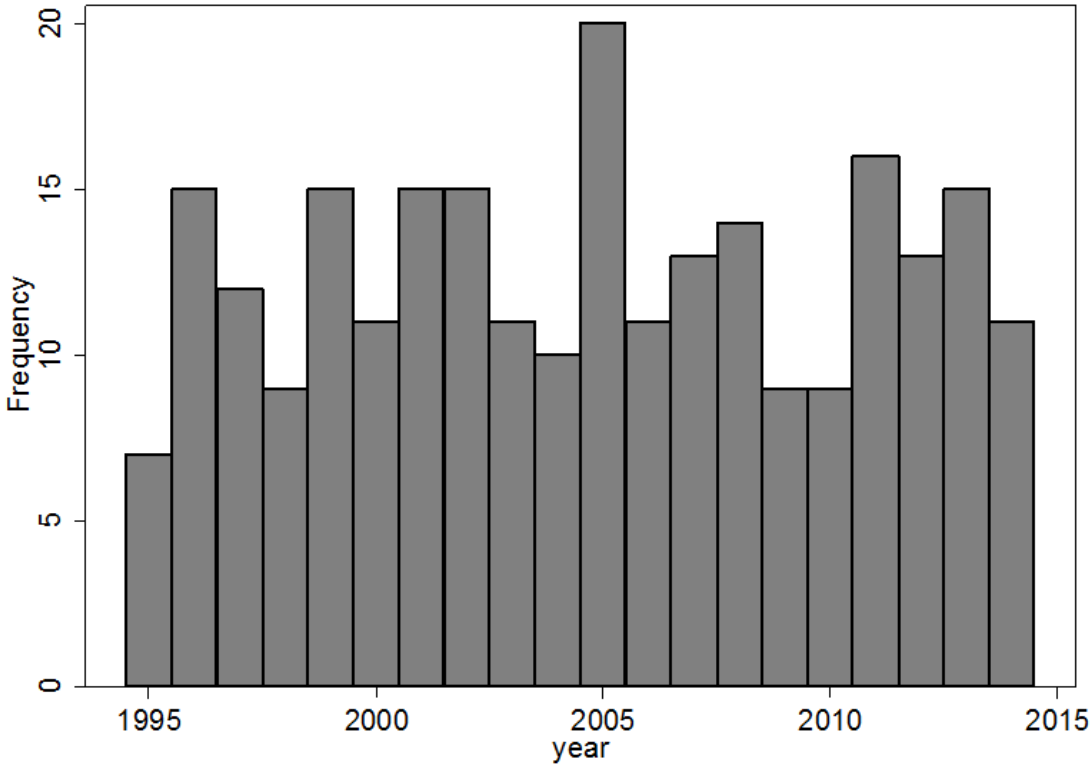

Figure 4-1: The frequency of submissions (251 total) of equine hepatic lesions by calendar year submitted to Prairie Diagnostic Services Inc. (Saskatoon, SK, Canada) between 1995 and 2014, inclusive.

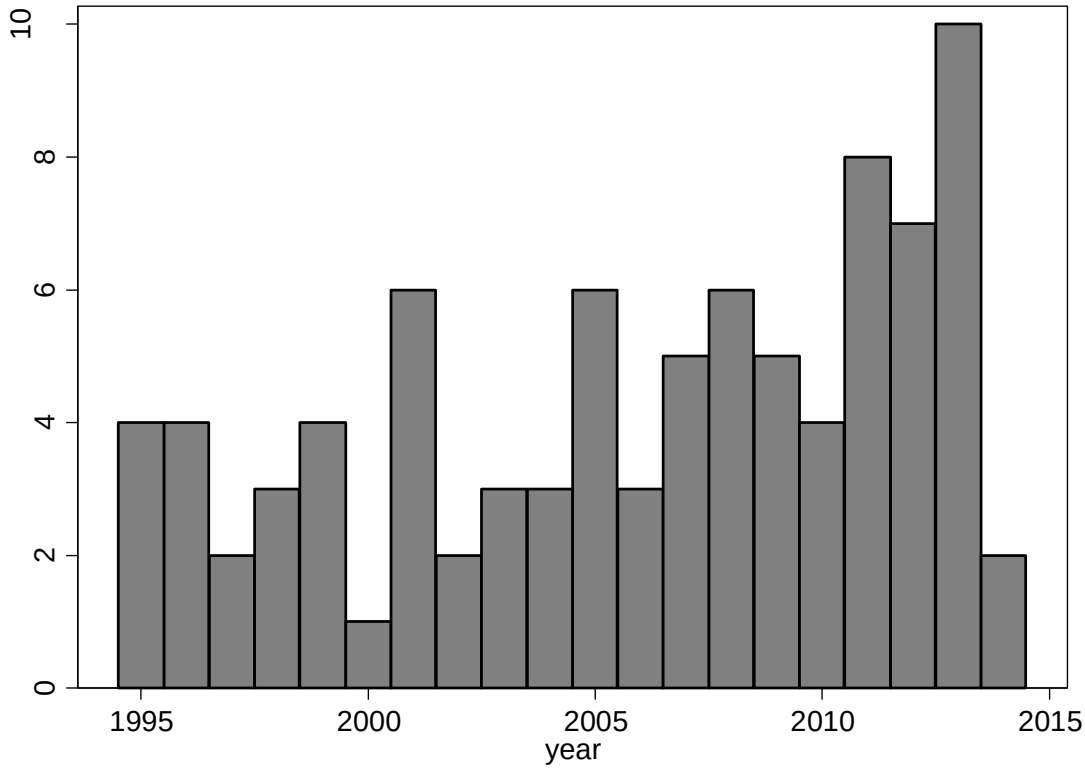

Figure 4-2: The frequency of submissions of suppurative to mixed hepatitis (88 total) in equine hepatic lesions by calendar year submitted to Prairie Diagnostic Services Inc. (Saskatoon, Canada) between 1995 and 2014, inclusive. Multi-variate analysis (logistic regression) determined that the odds ratio increase 1.07 times per year for this lesion (P = 0.005, 95% CI = 1.02 to 1.13) when life stage was combined in the model.

4.4.13 Results tables

Table 4-1. The frequency distribution of sex by life stage for all 251 equids with histopathologic hepatic lesions submitted to Prairie Diagnostic Services Inc. (Saskatoon, Canada), between 1995 and 2014, inclusive. Life stage was determined based on the given age of the animal, or from information from the submitted history or necropsy report if age was not specified. An adult horse was any animal over the age of 24 months, a yearling was 10 to 24 months (inclusive), and a juvenile (foal) was from birth to 9 months.

Sex	**Life stage**				
	Adult	**Yearling**	**Juvenile**	**Fetus**	**Total**
Male	77	9	23	5	114
Female	68	15	25	11	119
Unknown	7	0	2	9	18
Total	152	24	50	25	251

Table 4-2. The frequency distribution of breed by life-stage for all 251 equids with histopathologic hepatic lesions submitted to Prairie Diagnostic Services Inc. (Saskatoon, Canada), between 1995 and 2014, inclusive. Life stage was determined based on the given age of the animal, or from information from the submitted history or necropsy report if age was not specified. An adult horse was any animal over the age of 24 months, a yearling was 10 to 24 months (inclusive), and a juvenile (foal) was from birth to 9 months. Breeds were grouped as follows. Hotbloods included Thoroughbreds and similar breeds (including chuckwagon ponies), Arabians and Morgans. Warmbloods included Quarter Horses and similar breeds (including appaloosa, American paints, and pintos), Belgian Warmbloods and Foreign Warmbloods. Coldbloods included Percherons and Clydesdales. Ponies included all animals designated as "pony" as well as Welsh ponies and Shetland ponies. Donkeys included all donkeys and Miniature Donkeys. All cross- and mixed-breeds, two Standardbreds, one Saddle Horse, one Icelandic Horse and one Tennessee Walking Horse, were placed in the same category with the unknown breeds.

Breed	**Life stage**				
	Adult	**Yearling**	**Juvenile**	**Fetus**	**Total**
Hotbloods	**29**	**2**	**9**	**2**	**42**
Thoroughbreds	17	1	7	2	27
Arabian	9	1	2	0	12
Morgan	3	0	0	0	3
Warmbloods	**63**	**13**	**22**	**12**	**110**
Quarter Horses	58	10	19	9	96
Belgian Warmblood	5	2	2	0	9
Foreign Warmblood	0	1	1	3	5
Coldbloods	**2**	**1**	**2**	**2**	**7**
Ponies	**7**	**0**	**2**	**0**	**9**
Donkeys	**8**	**1**	**1**	**0**	**10**
Miniature Horses	**7**	**2**	**1**	**1**	**11**
Mixed/unknown	**36**	**5**	**13**	**8**	**62**
Total	**152**	**24**	**50**	**25**	**251**

Table 4-3. The frequency distribution (and percent proportion) of histopathologic lesion diagnosis by life stage for all 251 equids with hepatic lesions submitted to Prairie Diagnostic Services Inc. (Saskatoon, Canada), between 1995 and 2014, inclusive. Life stage was determined based on the given age of the animal, or from information from the submitted history or necropsy report if age was not specified. An adult horse was any animal over the age of 24 months, a yearling was 10 to 24 months (inclusive), and a juvenile (foal) was from birth to 9 months. Multi- and uni-variate analysis were performed to determine if life stage was a significant predictor for that lesion. The statistically significant life stages for particular lesion diagnoses are denoted by a ** or * if determined by multi-variate or univariate analyses, respectively.

Lesion	Life stage				
	Adults (%)	**Yearling (%)**	**Juvenile. (%)**	**Fetus (%)**	**Total (%)**
All lesions	152	24	50	25	251
Suppurative to mixed hepatitis	46 (30.3%)	13 (54.2%)**	26 (49.1%)**	3 (12.0%)	88 (35.1%)
Multi-focal necrosis	6 (3.9%)	1 (4.2%)	10 (20.0%)*	19 (76.0%)*	36 (14.3%)
Portal fibrosis with bile duct proliferation	28 (18.4%)*	2 (8.3%)	2 (4.0%)	-	32 (12.8%)
Hepatocellular vacuolation	20 (13.2%)	2 (8.3%)	3 (6.0%)	-	25 (10.0%)
Centrilobular necrosis	13 (8.6%)	3 (12.5%)	5 (10.0%)	3 (12.0%)	24 (9.6%)
Non-specific portal hepatitis (lymphocytic)	12 (7.9%)	1 (4.2%)	2 (4.0%)	-	15 (6.0%)
Granulomatous/ eosinophilic hepatitis	12 (7.9%)	2 (8.3%)	1 (2.0%)	-	15 (6.0%)
Neoplasia	12 (7.9%)*	-	-	-	12 (4.8%)
Infarction (focal necrosis)	3 (2.0%)	-	-	-	3 (1.2%)
Microvascular dysplasia (congenital)	-	-	1 (2.0%)	-	1 (0.4%)
Multi-focal eosinophilic granulomas	1 (0.7%)	-	-	-	1 (0.4%)

Table 4-4. The frequency distribution of breed by life-stage for all 251 equids with histopathologic hepatic lesions submitted to Prairie Diagnostic Services Inc. (Saskatoon, Canada), between 1995 and 2014, inclusive. From the date of the pathology report, year, and season of submission were determined. "Spring" was defined as the months of March, April, and May, "summer" as the months of June, July and August, "fall" was defined as September, October, and November, and "winter" was defined as December, January, and February.

Lesion	Season			
	Spring	**Summer**	**Fall**	**Winter**
Suppurative to mixed hepatitis	21	21	28	18
Multi-focal necrosis	20	7	2	7
Portal fibrosis with bile duct proliferation	6	9	11	6
Hepatocellular vacuolation	6	7	6	6
Centrilobular necrosis	6	6	5	7
Non-specific portal lymphocytic infiltrates	3	4	4	4
Granulomatous/eosinophilic hepatitis	2	7	3	3
Neoplasia	1	2	6	3
Infarction (focal necrosis)	0	1	2	0
Microvascular dysplasia	0	0	1	0
Multi-focal eosinophilic granulomas	0	0	1	0
Total	65	64	68	54

Table 4-5. Bacterial culture results of equine liver from submissions of suppurative to mixed hepatitis submitted to Prairie Diagnostic Services Inc. (Saskatoon, Canada), between 1995 and 2014, inclusive. Significant bacterial isolates are those known to cause hepatic lesions in equids.

	Submissions cultured	**Submissions with negative culture**	**Submissions with significant isolates**	**Significant isolates identified**
Acute cholangio-hepatitis	3	0	3	*S. equi* subsp. *zooepidemicus* *A. equuli, E. coli*
Chronic cholangio-hepatitis	8	5	1	*E. coli* (few)
Acute multi-focal hepatitis	10	6	4	*Listeria sp., Salmonella sp., S. equi* subsp. *zooepidemicus, A. equuli, E. coli.*

4.5 Discussion

We performed a 20-year retrospective study of histopathologic liver lesions in equids, including fetuses, based on cases submitted to PDS Inc, a diagnostic lab serving the public in western Canada. Lesions of suppurative (to mixed) hepatitis, multi-focal necrosis, portal fibrosis and bile duct proliferation and hepatocellular vacuolation were the most common lesions described and constituted almost 75% of all submissions. Lesions of centrilobular necrosis, non-specific portal lymphocytic infiltrates, granulomatous hepatitis, neoplasia, and infarction were less common. Single submissions of microvascular dysplasia and multi-systemic epitheliotropic eosinophilic disease were diagnosed. Statistics were performed to identify possible characteristics associated with the diagnosis of these lesions. Life stage was consistently associated with several lesion diagnoses whereas sex, season and collection method were not. Breed was significantly associated with only one lesion diagnosis, hepatocellular vacuolation.

Age and season are known to be associated with specific lesions or etiologies within the equine liver. For example, Tyzzer's disease and EHV-1 are known to affect neonatal foals, and EHV-1 is known to be an important cause of equine abortion.[108,178,270,273] Both etiologies often coincide with foaling season. In this study, age and lesion association in the equine liver were consistent with what has been reported in the literature. In general, lesions suggestive of infectious agents were more commonly reported in younger animals, and fibrosis was more commonly reported in older animals.

Suppurative to mixed hepatitis and multi-focal necrosis were caused by a variety of etiologies in this study and together made up nearly half of all submissions. They were also the most frequent lesions observed in fetuses and juveniles. Suppurative to mixed hepatitis commonly occurs secondary to a variety of bacterial infections. It is interesting to note that the number of submissions receiving this diagnosis steadily increased over the study period. This is most likely due to an actual increase in the incidence of this lesion, as the overall number of submissions of hepatic lesions per year has remained steady over the study period (except a small peak in 2005). Thus, pathologists and practitioners are encouraged to continue culturing liver (biopsy)

submissions whenever possible and to monitor for any changes in bacterial species and antibiotic resistance. Unfortunately, over 76% of the submitted specimens in this category were not cultured and over half of those cultured yielded negative or insignificant results. In many instances, only formalin-fixed biopsy or portion submissions were received. Therefore, the majority of cases in this category were without a specific bacterial cause although bacterial sepsis and ascending bacterial infections were often speculated in the pathology reports. It is often difficult to obtain positive culture results from liver samples submitted by veterinarians as they are commonly performed after administered antibiotics fail to achieve significant improvement. Pathologists are, therefore, reminded to submit liver samples from necropsies for bacterial culture more often. Bacterial culture of liver samples is not only important in determining the etiology of hepatic lesions but could also guide practitioners in selecting an appropriate antibiotic(s).

Significant bacterial isolates cultured from the liver in this study included *E. coli, Salmonella sp., Listeria sp., Actinobacillus equuli* and *Streptococcus equi* subsp. *zooepidemicus.* These isolates are consistent with what has been reported previously in horses and are considered enteric or septicemic in origin.[4,52,68,144,182,222] A few of the submissions of hepatitis in the current study also had concurrent gastrointestinal findings on post-mortem examination, ranging from enteritis, impaction, and obstruction caused by a high parasite burden. It has been shown that horses with gastrointestinal disease had significantly elevated serum hepatic enzyme activity, suggestive of hepatic injury.[67] Suppurative cholangiohepatitis has been associated with proximal enteritis in horses of one study.[68] Secondary hepatic lesions due to a primary gastrointestinal process may be a result of hypoxia, ischemia, endotoxemia, biliary obstruction or ascending bacterial infection.[290] The most common cause of death in horses, second only to old age, is colic and gastrointestinal disease.[282] Mortality due to colic in a normal farm population was 0.7 deaths per 100 horse years, with a fatality rate of 6.7%.[282] Human patients with gastrointestinal disease such as inflammatory bowel disease and celiac disease frequently have concurrent hepatobiliary lesions.[316] Therefore, primary gastrointestinal disorders are likely major contributors to hepatic lesions, and ultimately hepatic disease in horses.

Septicemia is an important cause of illness and death in young animals, with mortality reported in up to 75% in cases.[164] A study out of Ireland found that foals in their first year of life had morbidity associated with an infectious etiology in 46.5% of cases.[103] Young animals are especially at risk, as their immune systems are still naive. Inadequate or late colostrum intake may decrease the number of immunoglobulins transferred between mare and neonate.[223] Furthermore, the antibody profile of neonates is different from adult horses,[249] making them more at risk for infectious agents to which adult horses are usually resistant. Bacterial sepsis may cause secondary lesions in the liver through changes in liver hemodynamics, by direct injury of the hepatocytes, or both.[312] Not only are bacteria themselves directly damaging to cells, but bacterial products and inflammatory mediators have also been shown to cause hepatic damage and inflammation.[316] In human autopsy studies of patients that died of sepsis, hepatic lesions included portal inflammation, cholangiohepatitis, hepatocellular apoptosis, lobular inflammation, and steatosis.[163] In this study, it is likely that many horses had septicemia, but this cannot be conclusively confirmed.

The most commonly identified causes of random multi-focal necrosis were EHV-1 infection, followed by *Clostridium piliforme,* and both etiologies produced characteristic histological lesions and had typical age correlations. Equid herpes virus-1 is an important cause of pregnancy loss in mares and was the leading cause of hepatic lesions in aborted fetuses in this study.[178,273] Typical lesions of EHV-1 infection in the liver include random foci of hepatocellular necrosis with eosinophilic intranuclear inclusion bodies.[56,61] Since the virus often results in abortion acutely during infection, suppurative inflammation is not a consistent feature of the lesion, though some neutrophils may be present. Of the cases affected by EHV-1, all fetuses (19 in total) had characteristic and easily identifiable intra-nuclear inclusion bodies while no inclusions were found in juveniles (total of five submissions). It is not uncommon for inclusion bodies to be absent with viral infections as they are often found only during the early stages of the disease.[61] It is likely that these neonatal foals had a longer duration of infection since they were likely infected *in utero* and survived for few days after birth. Although not statistically significant in the current study, EHV-1 was most often diagnosed in March and April, with fetuses being aborted between 210 to 330 days of gestation. This seasonality is similar to what others have

described in the literature.[178,258,273] This seasonality may reflect the more natural time for pregnancy and parturition in mares without manipulation of mare fertility, as often practiced to produce an early-season foal. Although no breed predisposition could be statistically identified, Tengelsen *et al.*,[273] reported that Standardbreds were twice as likely as other breeds to be affected by EHV-1. Differences in breed susceptibility may reflect regional differences in desired breeds and management practices.

Portal fibrosis and bile duct proliferation was a relatively common diagnosis representing 12.7% of all submissions. In almost half of the cases, the exact etiology could not be identified, and the remainder were suspected to be due to chronic exposure to hepatotoxins, such as pyrrolizidine alkaloid-containing plants, alsike clover, and aflatoxins. Because of the delay (weeks to months) in the onset of clinical signs after exposure to a hepatotoxin, the original cause of the toxicosis is often difficult to identify. [107] Therefore, diagnosis is predominantly made based on history and supporting histopathology. Furthermore, as histopathologic lesions are due to long-term exposure, it is not surprising that these lesions were found in more mature animals. Typical histopathologic lesions of portal fibrosis and biliary hyperplasia are consistent with pyrrolizidine alkaloid toxicosis, alsike clover toxicosis, and chronic aflatoxicosis.[3,63,203] Also, the histopathologic lesions of pyrrolizidine alkaloid toxicosis and aflatoxicosis may include the presence of megalocytosis.[3] All the cases in the current study contained evidence of portal fibrosis and biliary hyperplasia, in the absence of any significant inflammation. Only a single case described megalocytosis, suggesting that perhaps alsike clover toxicities, versus pyrrolizidine or aflatoxin, are more common in this region than initially thought. Indeed, Nation described a regional distribution of alsike clover-like hepatic pathology in Northwest Alberta, a region that is included in this study. [203]

In those submissions in which hepatocellular vacuolation was the dominant lesion, the majority were interpreted to be lipid by the examining pathologist, though only four submissions were confirmed with Oil Red O stain. Similar to other studies of hepatic lipidosis in equids, [104,139,199,200] Donkeys (and Miniature Donkeys), ponies (including Shetland and Welsh Ponies) and Miniature Horses had significantly increased odds of being diagnosed with this lesion. In

fact, of the seven donkeys and three Miniature Donkeys in this study, the only diagnosed lesion was that of hepatocellular vacuolation. Despite having only two Percherons (Coldblood group) with this lesion in the current study, statistical analysis showed that this breed is more likely to be affected by hepatic vacuolation. However, it is more probable that the vacuolation is of secondary nature and represents hepatic lipidosis as both horses likely had a negative energy balance. One of these Percherons was a submitted pregnant mare, and had lesions consistent with acute enteritis and sepsis, with *Salmonella sp.* isolated from the gastrointestinal tract and uterus. The second individual was a yearling, with extensive and severe bronchointerstitial pneumonia, suspicious for influenza A virus, though it was not positive when tested with IHC.

Hepatic lipidosis in equids is due to a negative plane of nutrition, which may be due to anorexia, pregnancy or lactation, or, more often, secondarily to a systemic disease such as parasitism, gastric impaction, septicemia, colitis, esophageal obstruction and pituitary adenoma.[200] In a study of 23 Miniature Horses, enterocolitis was the most common primary disease causing lipidosis.[199] In the current study, only one mare with hepatic vacuolation was reported to be pregnant, and one mare was potentially still nursing. Two submissions had concurrent pituitary adenomas and concomitant hirsutism. No additional attempts were made in this study to further characterize the nature of hepatocellular vacuolation, as few had confirmed lipid or glycogen vacuoles and reports of antemortem clinical signs and diagnostics could not be confirmed.

Centrilobular necrosis can be seen with conditions causing low oxygen tension such as heart failure, DIC, and severe acute anemia.[61] Centrilobular hepatocytes are also prone to necrosis because they contain the highest concentration of cytochrome p450 enzymes compared to other zones within the liver.[207] In this study, this lesion primarily occurred in association with heart failure although it was also diagnosed with neonatal isoerythrolysis. Pathology reports also speculated about toxin exposure, DIC, and severe pneumonia as potential causes.

Non-specific portal lymphocytic hepatitis (also known as reactive hepatitis) is characterized by the presence of few lymphocytes in portal areas. This can be considered a normal finding or could be related to the response of the liver to systemic illness.[166] Only 15 submissions were

identified in this study, which is likely an under-representation of the actual number of cases as not all pathologists report this as a significant lesion.

Granulomatous inflammation was an uncommon type of inflammatory lesion in this study. Granulomas are aggregates of histiocytes and occur as a result of chronic antigenic stimulation, often due to mycobacteria, fungi or parasites.[53] Granulomatous inflammation made up only 6.0% of the hepatic lesions observed, but occurs in between 2.4% and 15% of biopsy specimens in humans.[53] In equids, these lesions are thought to arise from parasite migration which is often considered an incidental finding at necropsy.[33,263] Parasites capable of causing these lesions in equids includes *Strongylus vulgaris, Strongylus equinus, Strongylus edentatus, Parascaris equorum and Habronema sp.*.[183,263,286] In this study, 13 cases were thought to be due to parasite migration, yet only one horse had cross sections of *Parascaris equorum* within the granulomatous nodules. In another study, only one of 11 horses had evidence of parasites within granulomatous lesions (schistosome egg remnants).[33] In general, however, most cases of parasitic hepatic lesions do not cause clinical disease, though in rare instances animals may succumb to liver failure, circulatory disturbances or poor body condition as a result.[33]

Primary hepatic neoplasia is rare in horses,[17] and cholangiocarcinoma was diagnosed only once in 12 cases of hepatic neoplasia in this study. The most diagnosed neoplasm in the equine liver in the current study was multicentric lymphoma, a relatively common neoplasm in horses. Only one case of metastatic SCC was diagnosed in this study and was of gastric origin. However, SCC was the most common malignant neoplasm in one study involving 241 equids, though the most common primary site was the bladder.[192] One case of hemangiosarcoma involving the liver was found in this study. The prevalence of hemangiosarcoma in horses ranges between 0%- 0.7% and the liver has been described as a site of metastasis.[96] Also, one case of renal cell carcinoma was found in the liver in the current study, and the lung and liver are known to be the most common sites of metastasis of renal cell carcinoma in equids.[157] In conclusion, though neoplasia is a relatively rare occurrence in horses, the prevalence and behavior in this study are in alignment with what is currently known.

Three cases of infarction and a single case each of congenital microvascular dysplasia and MEED were observed in the current study. Hepatic infarction is uncommon, and the low incidence in comparison to other organs has been ascribed to the unique characteristics of hepatic vascularization.[42] Two of the affected animals in the current study had infarctions due to a liver lobe torsion. Congenital microvascular dysplasia is a rare condition of young foals and infrequently reported in the literature.[209] One case of MEED, also in a Quarter Horse, has been described from the local equine population in Saskatchewan.[41] The particular animal was treated and survived and was, therefore, not included in this study. However, most equids do not survive the disease, despite treatment.[41]

In general, there is a good correlation between hepatic biopsy and necropsy findings.[275,303] In this study, biopsy submissions comprised only a small proportion of all collected specimens but were often able to detect the major lesions. Biopsy submissions of suppurative or granulomatous inflammation, fibrosis and bile duct proliferation, hepatocellular vacuolation, necrosis and diffuse neoplasia (lymphoma) were detected in this study. This is similar to what West described, where they reported that necrosis and fibrosis were reliably detected in biopsy submissions, though the cause of the lesion could not always be ascertained.[303]

Within the current study, some limitations were identified. These include the retrospective nature of the study, and the diverse nature of many lesions in this study, making them sometimes difficult to place into a respective lesion category. Some limitations of the statistical analysis were also identified. Statistical analysis often involved comparison of small groups, which may have limited the statistical power. This was the case for certain breed groups, despite combining like breeds together. Furthermore, the regional nature of this study may not exactly represent disease prevalence for the North American horse population as only submissions to the diagnostic service in Saskatoon were reviewed. Also, these submissions may not reflect the distribution of cases in a true population as submissions are dependant on factors such as the attitudes of owners and submitting practitioners, the distance to travel for a necropsy and the frequency of voluntarily donated cases. Lesion distribution in a true population is unknown. Therefore, all the other lesions collectively became the reference group for statistical analysis,

and this reference group likely does not reflect lesion prevalence in a true population. In addition, the statistics performed were done in comparison only to what was present within this submitted population. For example, statistical analysis of breed susceptibility to a lesion diagnosis was done in relation to the "unknown" breed group. Even though the "unknown" breed group likely contains the largest variety of breeds (mixed-, cross- and unknown-breeds), the distribution of breeds in the "unknown" group is not the same as one would expect in a true population.

Potential future work could involve a more prospective approach, by correlating the variety and severity of clinical signs with antemortem, serial blood evaluation (serum biochemistry and complete blood count) and biopsy results with survival times and post-mortem findings. Furthermore, it would be interesting to correlate lesions observed by ultrasonography, biopsy and post-mortem examination in an attempt to provide a more sensitive antemortem tool to diagnosing hepatic disease in horses.

This is the first retrospective study of hepatic lesions in equids from western Canada. Life stage was statistically associated with several lesion types, including suppurative to mixed hepatitis (juveniles, yearlings), portal fibrosis and bile duct proliferation (adults), centrilobular necrosis (juveniles, fetuses), and neoplasia (adults). Several breeds were significantly associated with hepatocellular vacuolation. Therefore, the findings in this study, though specific to a regional population in western Canada, are in alignment with what we currently know about disease in equids, suggesting that the regional population in this study shares similarities with the equine population in general, with regard to hepatic disease.

4.6 Acknowledgements

This study could not have been possible without the work of all the pathologists from both PDS, Inc. and the WCVM, that evaluated the cases over the 20-year study period.

Funding was kindly provided by the Townsend Equine Health Research Fund and the Western College of Veterinary Medicine Interprovincial Graduate Fellowship.

4.7 Supplemental Materials

Supplemental Table 4-1. The *P*-values for the test of significance for individual characteristics per lesion type by univariate analysis. Chi-square analysis was carried out for each lesion to determine if the tested characteristic (breed, life stage, sex, year of lesion diagnosis and season) was significantly associated with the occurrence of that lesion in comparison to all other lesion groups. Potential significant characteristics were identified at a relaxed level of significance ($P \leq 0.2$) and further evaluated by multi-variate analysis. The final model building included a more restrictive level of significance ($P \leq 0.05$). The P – value is the probability of there being no difference in the distribution of that diagnosed lesion by the tested characteristic.

Breeds were grouped together as follows. Hotbloods included Thoroughbreds and similar breeds (including chuckwagon ponies), Arabians and Morgans. Warmbloods included Quarter Horses and similar breeds (including appaloosa, American paints, and pintos), Belgian Warmbloods and Foreign Warmbloods. Coldbloods included Percherons and Clydesdales. Ponies included all animals designated as "pony" as well as Welsh ponies and Shetland ponies. Donkeys included all donkeys and Miniature Donkeys. All cross- and mixed-breeds, two Standardbreds, one Saddle Horse, one Icelandic Horse and one Tennessee Walking Horse, were placed in the same category with the unknown breeds. Life stage was determined based on the given age of the animal, or from information from the submitted history or necropsy report if age was not specified. An adult horse was any animal over the age of 24 months, a yearling was 10 to 24 months (inclusive), and a juvenile (foal) was from birth to 9 months.

Microvascular dysplasia and multi-focal eosinophilic granuloma lesions could not be tested as they were only represented by a single submission each.

(table on following page)

Lesion	Characteristic tested for significance					
	breed (grouped)	life stage	sex	year	season	collection method
Hepatitis (suppurative to mixed) (n = 88)	0.213	0.001	0.420	0.140	0.672	0.273
Multi-focal necrosis (n = 36)	0.392	0.000	0.001	0.877	0.000	0.058
Biliary fibrosis with bile duct proliferation (n = 32)	0.090	0.008	0.211	0.908	0.643	0.156
Hepatocellular vacuolation (n = 26)	0.000	0.113	0.299	0.566	0.894	0.562
Centrilobular necrosis (23)	0.345	0.826	0.906	0.868	0.718	0.157
Non-specific portal hepatitis (lymphocytic) (n = 15)	0.157	0.381	0.219	0.580	0.936	0.305
Granulomatous/ eosinophilic hepatitis (n = 15)	0.737	0.238	0.994	0.378	0.252	0.514
Neoplasia (n= 12)	0.480	0.042	0.919	0.627	0.221	0.311
Infarction (focal necrosis) (n = 2)	0.859	0.726	0.298	0.393	0.610	0.778

CHAPTER 5: INTRODUCTION TO CHAPTER 6

The previous study (chapter 4) investigated the types of lesions in equids from western Canada. In general, the diagnosis and treatment of hepatic disease in equids is often delayed as clinical signs are vague and occur after a significant loss of hepatic function. Treatment is mainly only supportive.

Metallothionein (MT) has been investigated in extensively in human hepatic disease, especially as a possible treatment for chronic fibrotic diseases. The following chapter investigates the potential role of MT in chronic hepatic lesions in equids. Ultimately, can MT be utilized as a possible treatment for chronic equine liver disease, independent of the etiology? As the results of the previous study were in alignment with what we understand about liver disease in equids from the literature, the results of the MT study may also apply to the equine population in general.

CHAPTER 6: METALLOTHIONEIN EXPRESSION IS RELATED TO KI-67 IMMUNOREACTIVITY WITHIN BILE DUCT EPITHELIUM AND PARENCHYMAL INFLAMMATORY CELLS IN EQUINE LIVER DISEASE

This chapter contains the complete text of a manuscript submitted for publication (currently under revision) to *Veterinary Pathology*.

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6.1 Abstract

Chronic liver disease is an important cause of illness in horses and may result in mortality in up to 25% of affected animals. Metallothionein (MT) is a highly conserved intracellular protein with a high binding affinity for divalent cations that has been shown to play a major role in inflammation and cellular regeneration. This study aimed to evaluate the role of MT in horses affected by chronic hepatic lesions by evaluating the relationship between hepatocyte MT expression, assessed by immunohistochemistry (IHC), and the degree of inflammation, fibrosis and bile duct proliferation in 77 selected cases. In addition, the proliferation of hepatocytes, bile duct epithelium, and inflammatory cells was determined using IHC for Ki-67, a protein expressed during all active stages of the cell cycle. Inflammation and fibrosis were given scores from 0 to 3 depending on severity and bile duct proliferation was assessed as the presence or absence of proliferation, with four bile ducts per field being the cut-off value. Increased MT expression was observed in 73 of 77 (94.8%) cases. Ki-67 expression was seen in resident Kupffer cells (42/77 cases, 54.6%), bile duct epithelium (10/77 cases, 13.0%), and hepatocytes (8/77 cases, 10.4%). Median MT expression was higher in cases containing lymphocytic infiltrates compared to cases with no lymphocytic infiltrate ($P < 0.05$) and all foci showed Ki-67 staining lymphocytes (39/77 cases, 50.7%), Metallothionein expression was also significantly associated with Ki-67 staining in bile duct epithelium and Kupffer cells. These results suggest a putative role for MT during liver inflammation and proliferation of bile duct epithelia in horses.

Key Words: bile duct, equine, fibrosis, immunohistochemistry, Kupffer cells, liver, lymphocytes, metallothionein.

6.2 Introduction

Liver disease is an important cause of illness in horses. It affects all ages, breeds, and sexes and may result in fatality in 25% of cases.[87] Causes of chronic hepatic disease in horses include aflatoxicosis,[63] pyrrolizidine alkaloid toxicity,[274] and cholelithiasis,[144] among others. Because of the lack of specificity in clinical signs, liver disease diagnosis is largely based on biopsy and serum biochemical analyses.[19] In general, most signs of hepatic failure appear suddenly, regardless of the cause and duration of the underlying disease, after the loss of more than 75% of the functional capacity of the liver.[19] Irrespective of the cause, the prognosis of hepatic disease depends on the severity of histological lesions. Moderate or severe hepatic fibrosis has been associated with significantly higher mortality rates.[87] Furthermore, the exact etiology of chronic liver disease in horses is rarely identified, which limits treatment options.

Metallothionein (MT) was first identified in 1957 as a cadmium- and zinc-binding protein produced in the equine renal cortex.[147] It is a low molecular weight, highly conserved intracellular protein with high binding affinity for divalent cations, especially copper, cadmium, and zinc.[277] Virtually all eukaryotic organisms, as well as some prokaryotic organisms, express MT.[214] Four major isoforms of MT exist in mammals (MT-1, -2, -3 and -4), of which MT-1 and -2 are most abundant in mammalian cells and are considered a single MT due to their high homology.[215,278] Most mammalian cells express low levels of MT, but there is tissue specificity to expression. Liver MT is predominantly associated with copper and zinc, whereas kidney MT binds mainly copper, cadmium, and zinc.[48]

Metallothionein is typically found in the cytosol of resting, non-proliferating cells, but can be translocated to the nucleus during cell proliferation and differentiation.[3] Intranuclear localization of MT is thought to be associated with gene expression during the cell cycle, as a mechanism to protect DNA from damage and cell apoptosis.[47] Elevated levels of intra-nuclear MT may be related to an increased demand for zinc during rapid growth as zinc is required for transcription factors and enzyme function.[47] Furthermore, the presence of MT within the nucleus of human neoplastic cells is indicative of their mitotic activity, as MT immunostaining was most intense at

the proliferative edge of malignant tumors.[46] In addition, a four-fold increase in cellular MT levels was measured in proliferating liver cells when compared to MT levels in resting cells.[264] MT's role in carcinogenesis has also been suggested, and *in vitro* studies have postulated that p53 and the estrogen-receptor may be involved in the induction of MT in human neoplastic epithelial cells.[48]

Metallothionein gene expression can be induced by various hepatotoxic molecules such as heavy metals, carbon tetrachloride, and ethanol, as well as certain cell stressors including cellular starvation and hydric stress.[277] Studies have shown that it may play a major role as an anti-inflammatory agent.[132,133] For example, MT appears to be protective against lipopolysaccharide (LPS)-induced acute lung inflammation when compared to MT-knock out mice.[271] Several pro-inflammatory cytokines, including interleukin-6 (IL-6), tumor necrosis factor-α (TNFα) and interferon-γ (IFNγ) have been shown to induce the expression of MT.[70] The MT gene contains several response elements that upregulate its transcription, including glucocorticoid response elements, metal response elements, and an anti-oxidant response element.[69]

Despite strong evidence of the involvement of MT in inflammation, cell regeneration and neoplasia, 50 years of research has not yet delivered any definitive knowledge on MT's function,[214] though its highly conserved structure and widespread prevalence suggests it has an important evolutionary role. It has been the topic of several review papers.[57,214,278]

Recent work by Sridharan et. al.[262] has shown an interesting, positive correlation between MT expression and hepatocyte regeneration and inflammation in chronic liver disease in dogs. With the growing evidence that MT plays a role in hepatic inflammation and fibrosis, the present study aimed to explore its role as a defense mechanism in chronic hepatic disease of horses. This was accomplished by evaluating the relationship between hepatocyte MT expression by immunohistochemistry and the degree of hepatic inflammation, fibrosis, bile duct proliferation, and hepatocellular proliferation in cases of equine liver disease.

6.3 Material and Methods

6.3.1 Case selection and histological scoring

Pathology reports from the archives at Prairie Diagnostic Services, Inc. (PDS). (Saskatoon, SK, Canada) between January 1, 1995, and December 31, 2014, were reviewed for cases of chronic equine liver disease by the presence of fibrosis (JNCV). These cases were then reviewed by hematoxylin and eosin (H&E) slide evaluation and were included in the study if they contained at least minimal fibrosis, with or without inflammation or bile duct proliferation (JNCV and ANA). Tissues were included in the study if a minimum of 1 cm^2 was available for assessment and no autolysis was seen histologically.

In each section, hepatic inflammation, fibrosis, and bile duct proliferation were scored by two independent pathologists (ALA and ANA) using a grading scheme modified after Sridharan et al. (Figs. 4-1 to 4-9).[262] Hepatic inflammation was subjectively scored as 0, 1, 2, or 3 based on the assessment of the entire H&E section. A score of 0 indicated the absence of inflammation, a score of 1 indicated the presence of inflammation in < 10% of the section, a score of 2 indicated the presence of inflammation in 11% to 50% of the section and a score of 3 indicated > 50% of the section was affected. Similarly, fibrosis was scored 0, 1, 2 or 3 based on the assessment of the same sections stained with Masson's Trichrome stain. A score of 0 indicated the absence of fibrosis, a score of 1 indicated the presence of periportal fibrosis, a score of 2 indicated the presence of fibrosis extending away from portal tracts into the parenchyma, and a score of 3 indicated the presence of bridging fibrosis. Bile duct proliferation was evaluated by counting the number of bile duct profiles in 5 arbitrary fields that contained at least one bile duct, at 400x magnification. The number of bile ducts counted per pathologist was then averaged, and the cases were subsequently divided into two groups based on the presence or absence of bile duct proliferation to improve statistical power. Cases with greater than 4 bile ducts per field were considered to have bile duct proliferation, whereas cases with 0 to 4 bile ducts per field were regarded as not proliferated. The cut-off value of four bile ducts per field was determined by utilizing the bile duct counts of six histologically normal liver sections (ten random fields per section) measured by both pathologists. Four or fewer bile ducts per field were found in 95% of

the 60 counted fields.

6.3.2 Immunohistochemistry for metallothionein and Ki-67

Immunohistochemistry for MT and Ki-67 was performed at the PDS laboratory using the following protocol, as previously described (with modifications).[118,231] Consecutive 5 μm sections from formalin-fixed, paraffin embedded tissue blocks were cut and mounted onto charged slides (ProbeOn Plus or SuperFrost Plus, Fisher) and oven baked at 60° C for 60 minutes. Tissue sections were subsequently deparaffinized in xylene for 5 minutes, followed by rehydration in successive graded ethanol (5 minutes each: 100%, 95%, 70% ethanol) and then rinsed in distilled water. The tissue sections were then placed in 3% hydrogen peroxide solution (in methanol) for 10 minutes, to block any non-specific endogenous peroxidase activity and then rinsed twice in phosphate buffered saline with Tween 20 (PBST) for 5 minutes. Heat-induced antigen retrieval was performed in a water bath (Dako PT Link) at 97° C in Tris/ethylenediaminetetraacetic acid (EDTA) buffer (pH 9) for 20 minutes and allowed to cool in IHC buffer.

The MT primary antibody (clone E9, mouse anti-horse MT-1 and MT-2, monoclonal IgG1, Dako) was diluted 1:1000 in antibody diluent (Dako) and incubated on the slides for 30 minutes at room temperature. Incubation for the Ki-67 primary antibody (clone MIB-1, mouse anti-human, monoclonal IgG1, Dako) was performed overnight at 4° C and with a 1:50 dilution in antibody diluent. After incubation with primary antibody had been completed, slides were rinsed 3 times for 5 minutes with PBST and incubated with Dako Envision+ System. Color development was performed using diaminobenzidine (DAB) substrate buffer and DAB chromagen as per manufacturer's instructions. Slides were counter-stained with hematoxylin at room temperature for 4 minutes, rinsed with water and mounted with coverslips.

Six histologically normal liver samples from horses obtained during necropsy were stained for MT and Ki-67 expression. Positive control samples for MT staining included equine skin and kidney as previously described.[160,252] Positive control samples for Ki-67 staining included equine duodenum with Peyer's patches. Appropriate negative sample controls were performed by

omission of the primary antibody, as well as by substitution of the primary antibody by an irrelevant monoclonal IgG antibody. (MT protocol development was performed by JNCV).

6.3.3 Scoring of hepatic metallothionein and Ki-67 expression

Metallothionein expression in hepatocytes was evaluated by counting the number of positive cells utilizing an intra-ocular grid at 400x measuring 25 μm^2 (JNCV). Ten fields were counted in each section in a diagonal square zig-zag pattern, and a mean number per field was calculated. A mean of 104.5 hepatocytes was counted within the 25 μm^2 grid. Ki-67 expression was evaluated by noting the presence or absence of nuclear staining within hepatocytes, Kupffer cells, biliary epithelium and any lymphocytic infiltrates within the entire liver section(s) (JNCV).

6.3.4 Rhodanine staining for copper

Ten slides with elevated MT expression were stained with rhodanine stain as previously described.[248] Copper content was assessed subjectively by evaluating the slides with light microscopy (JNCV).

6.3.5 Statistical analysis

Statistical analysis was performed using Stata 14.2 (StataCorp, College Station, Texas, USA) (JCSH and JNCV). Inter-rater agreement between the pathologists was determined by calculating a *kappa* score for each of inflammation, fibrosis and bile duct proliferation. The *kappa* score was interpreted according to Landis and Koch.[167] A Spearman's Rank test was used to determine the presence of correlation between inflammation, fibrosis, and bile duct proliferation. The data on MT expression was not normally distributed. Therefore, non-parametric statistics were used. A Dunn's test with a post-hoc Sidak adjustment (to account for multiple comparisons) was used for comparing group differences in hepatocyte MT expression by inflammation score (0 to 3) and fibrosis score (0 to 3) determined by each pathologist separately. A Mann-Whitney U-test was used to assess potential differences in hepatocyte MT expression by nuclear Ki-67 expression (presence/absence) within hepatocytes, bile duct epithelium, intra-sinusoidal Kupffer cells and lymphocytic infiltrates. Additionally, a Mann-Whitney U-test was used to assess potential differences in hepatocyte MT expression by the presence or absence of bile duct proliferation

determined by each pathologist separately. The six histologically normal liver samples were not included in the statistical analysis. In all analyses, the results were considered statistically significant when $P < 0.05$.

6.3.6 Methods Figures

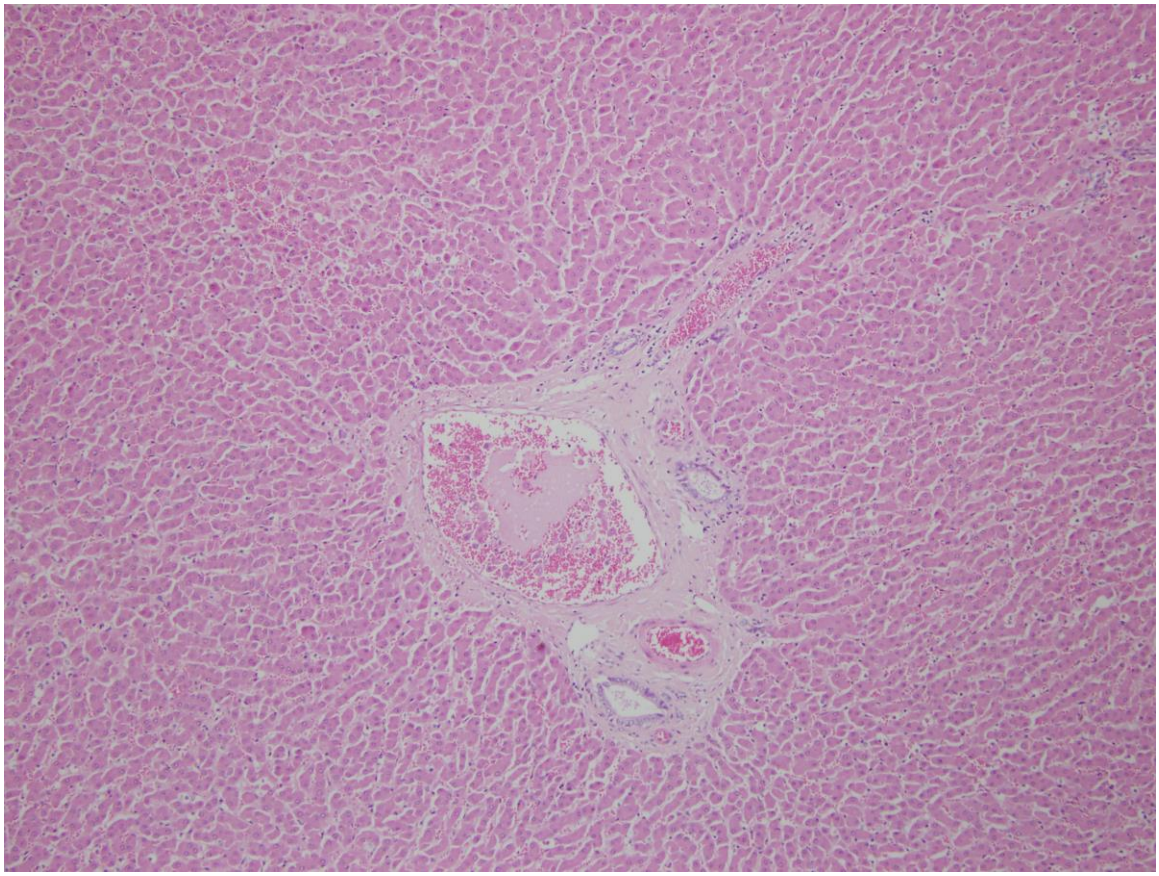

Figure 6-1: Equine liver. A representative image of a histologically normal liver section (hematoxylin & eosin, 100x). There is no evidence of inflammation present (inflammation score 0), and no bile duct proliferation is observed.

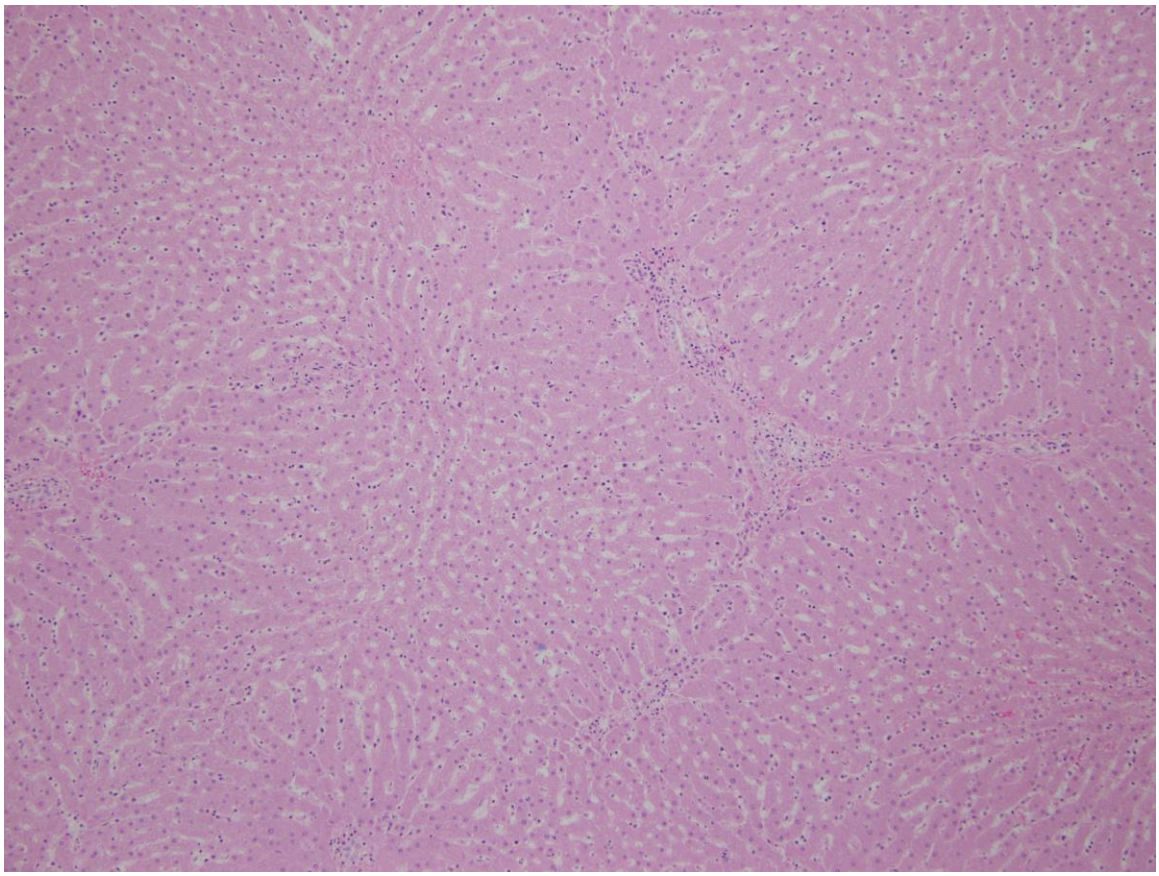

Figure 6-2: Diseased equine liver. Scoring system for equine hepatic disease, inflammation score 1. There is mild inflammation occupying < 10% of the section. (hematoxylin & eosin, 100x).

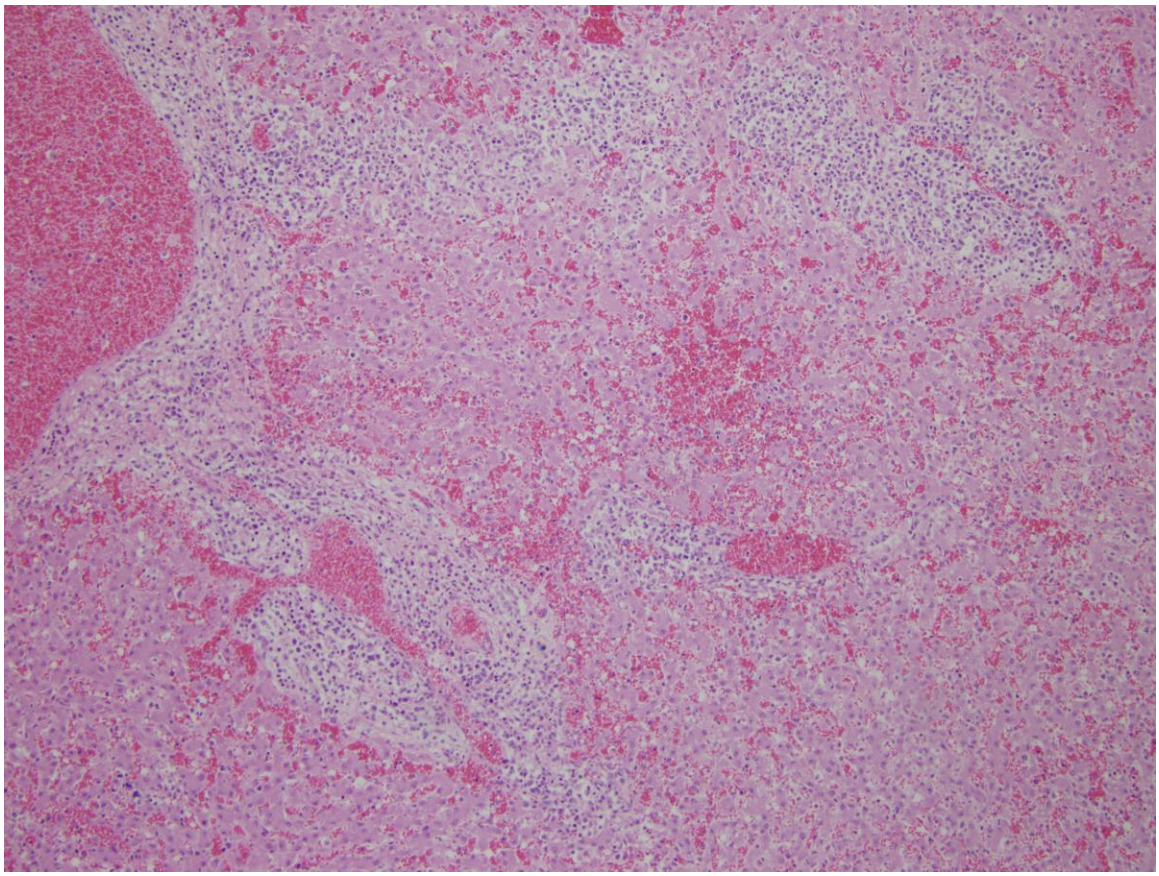

Figure 6-3: Diseased equine liver. Scoring system for equine hepatic disease, inflammation score 2. Approximately 11% to 50% of the section is affected by inflammation (hematoxylin & eosin, 100x).

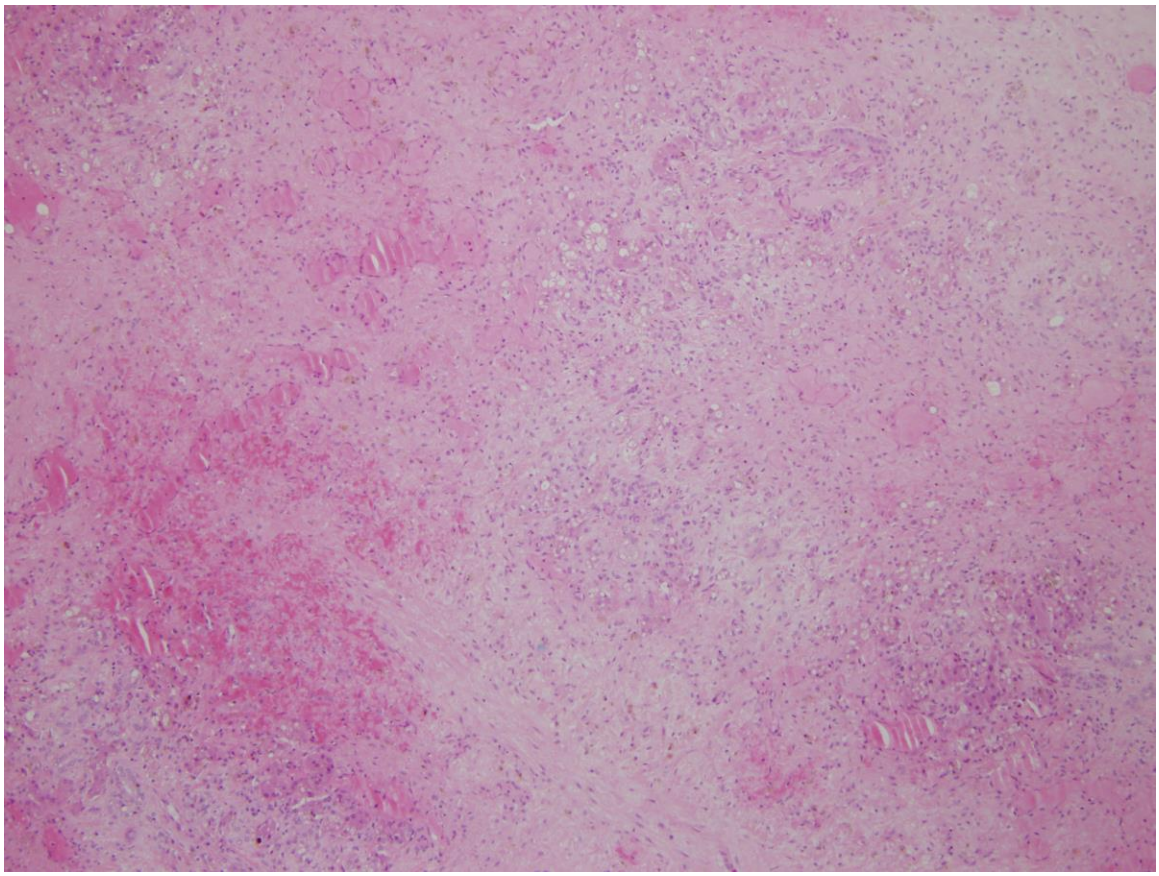

Figure 6-4: Diseased equine liver. Scoring system for equine hepatic disease, inflammation score 3. Greater than 50% of the section is affected by inflammation. (hematoxylin & eosin, 100x).

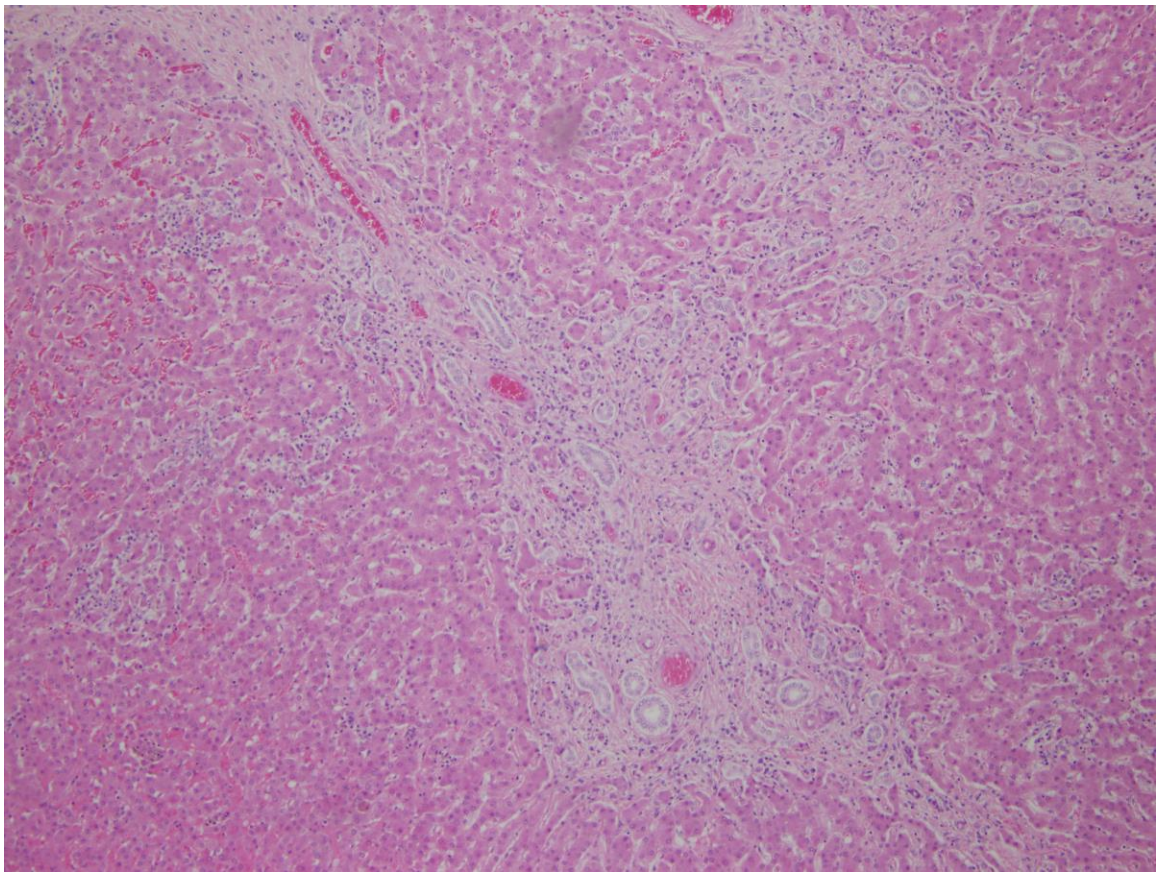

Figure 6-5: Diseased equine liver. Scoring system for equine hepatic disease, bile duct proliferation. Multiple bile duct profiles are evident in this photomicrograph. (hematoxylin & eosin, 100x).

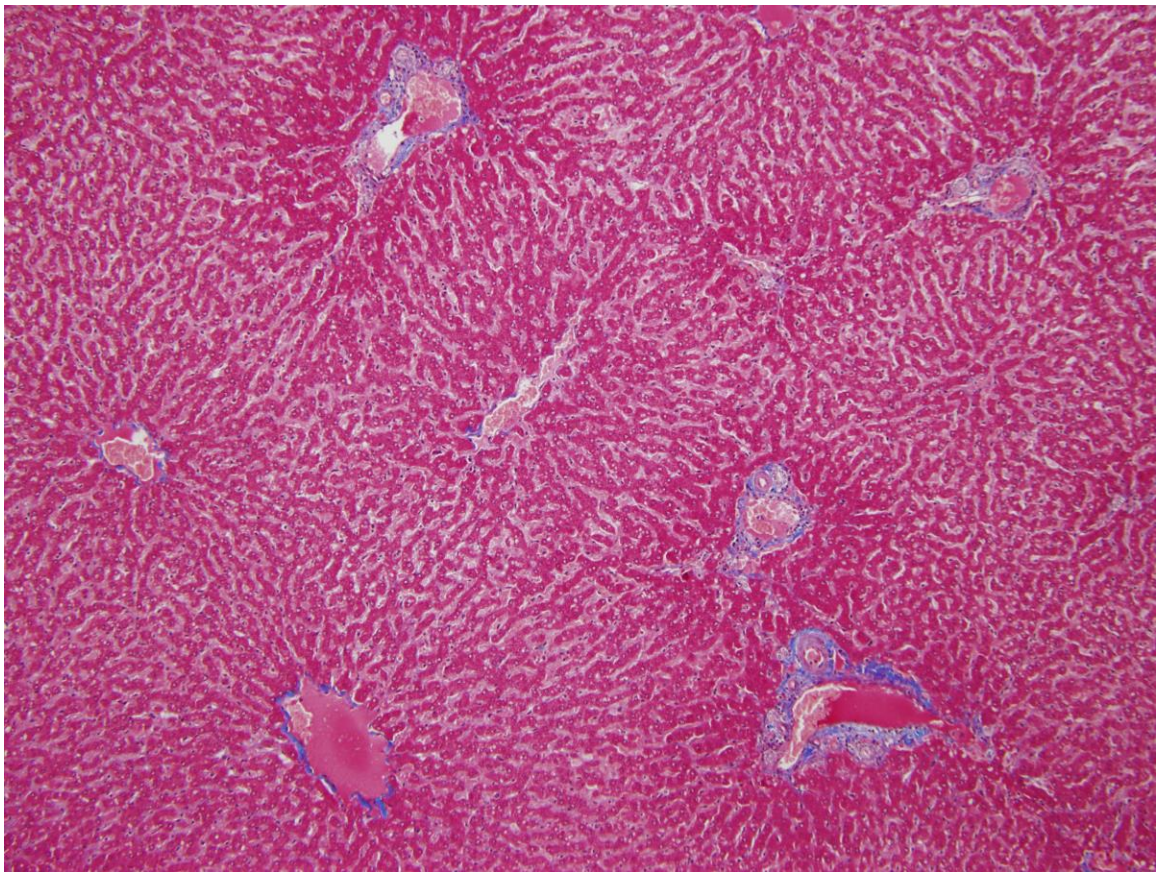

Figure 6-6: Normal equine liver. Scoring system for equine hepatic disease, fibrosis score 0. (Masson's trichrome stain, 100x).

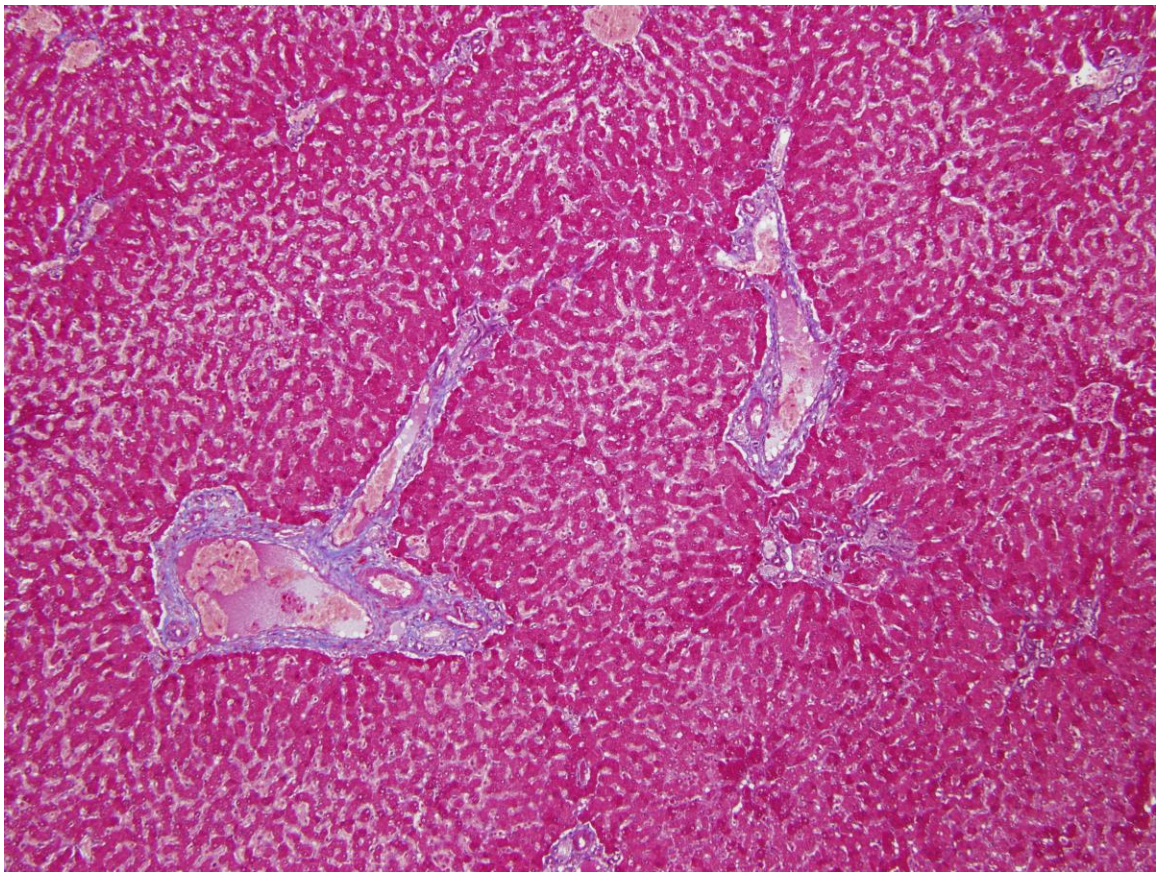

Figure 6-7: Diseased equine liver. Scoring system for equine hepatic disease, fibrosis score 1. Mild increase in portal collagen. (Masson's trichrome stain, 100x).

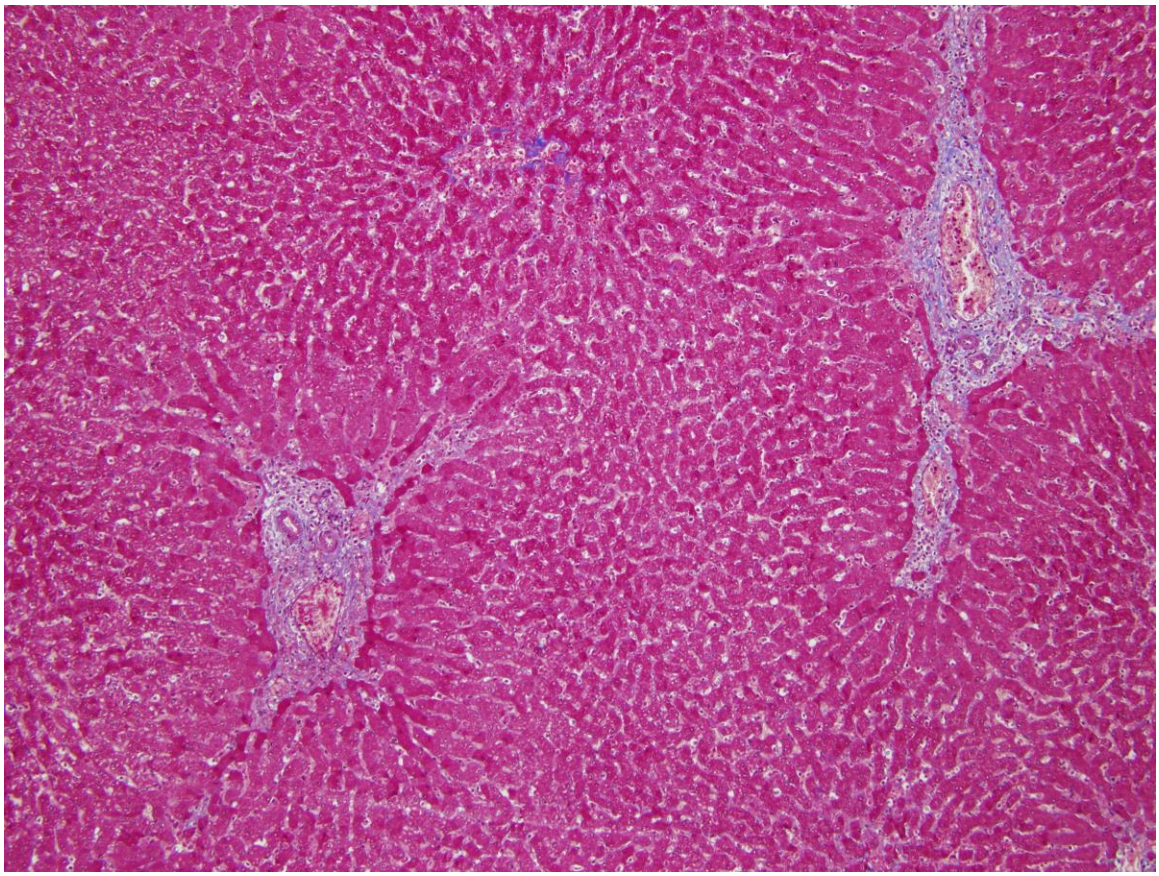

Figure 6-8: Diseased equine liver. Scoring system for equine hepatic disease, fibrosis score 2. There is non-bridging fibrosis within the hepatic parenchyma. (Masson's trichrome stain, 100x).

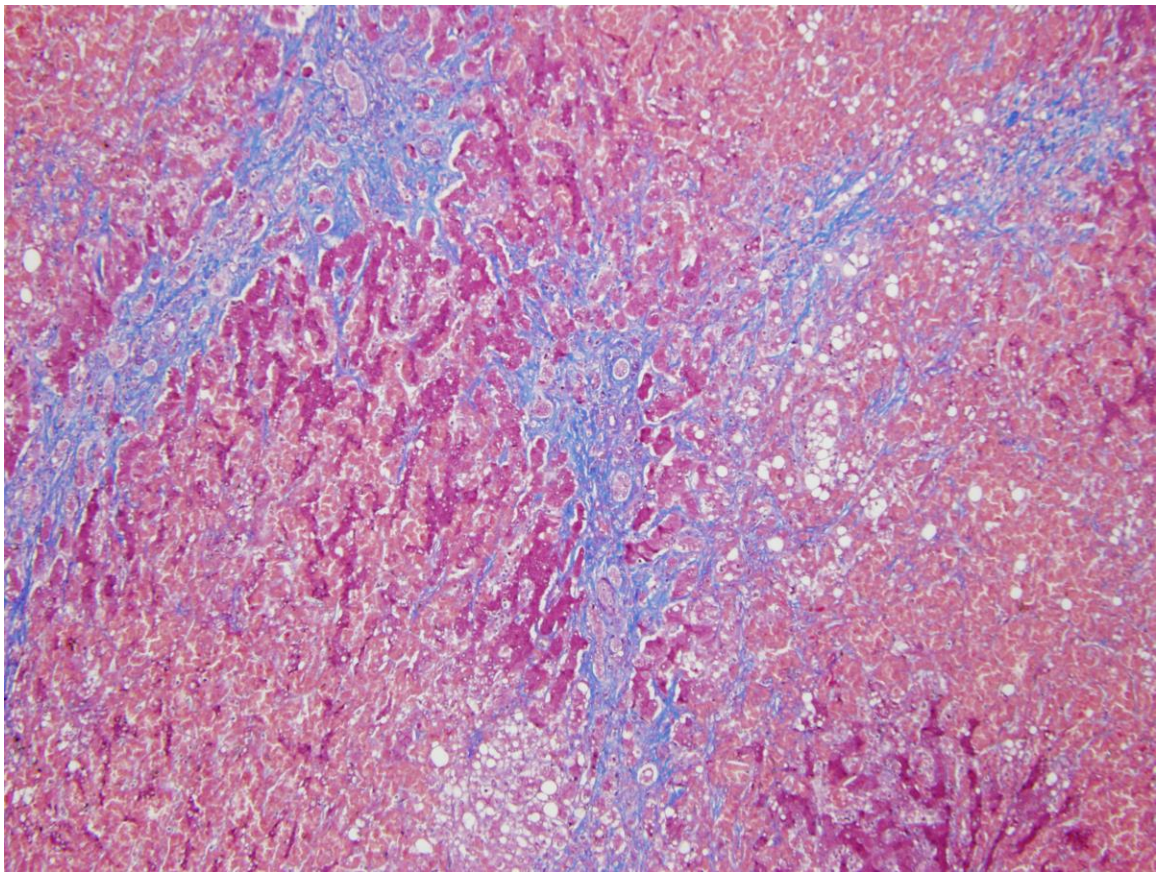

Figure 6-9: Diseased equine liver. Scoring system for equine hepatic disease, fibrosis score 3. There is extensive bridging fibrosis within the hepatic parenchyma. (Masson's trichrome stain, 100x).

6.4 Results

The kappa statistic determined that a moderate agreement was found between the two pathologists who independently examined all cases for each of the evaluated parameters, as interpreted according to Landis and Koch (Table 4-1).[167] Inflammation was present in 81.8% of cases, and bile duct proliferation was present in 35.7% of cases. A positive correlation was observed between inflammation and fibrosis scores using Spearman's Rank Test (Spearman's rho = 0.4046, $P = 0.003$).[54] No significant correlation was found between bile duct proliferation and inflammation or fibrosis.

The expression of MT and Ki-67 in normal liver samples was low to absent in comparison to diseased livers (Figs 4-10 and 4-11, respectively). Metallothionein expression was observed within hepatocytes in 73 out of 77 (94.8%) cases of equine liver disease, with a median of 42.0 positive cells and a range of 0 to 166.3 positive cells per 25 μm^2. Both cytoplasmic and nuclear staining was observed. Metallothionein expression was also occasionally observed in bile duct epithelium and lymphocytes.

Out of 77 cases, 8 (10.4%) had evidence of Ki-67 expression within hepatocyte nuclei (Fig. 4-12), 10 (13%) had nuclear expression within bile duct epithelium (Fig. 4-13), and 42 (54.6%) displayed nuclear expression within Kupffer cells (Fig. 4-14). Nuclear staining was more prominent within bile duct epithelial cells than hepatocytes, where the pattern of expression was sparse and limited to individual random hepatocytes. Strong Ki-67 expression was found in all lymphocytic inflammatory foci (Fig. 4-14).

Rhodanine staining for intracellular copper within hepatocytes was seen in 3 out of 10 cases of diseased liver. The distribution of positive rhodanine staining was random, sparse and observed only within individual or small aggregates of cells, predominantly in periportal areas (Fig. 4-15). The staining intensity within hepatocytes of normal liver samples was very weak to absent.

Metallothionein expression was significantly associated with Ki-67 staining in bile duct epithelium and Kupffer cells (Table 4-3). More specifically, median MT expression within hepatocytes was significantly increased when Ki-67 expression was observed within bile duct epithelium, in contrast to when it was absent (median 106.8 versus 33.0 cells/25 μm^2, P = 0.0004; Fig. 4-17). In addition, when MT expression was compared between the presence or absence of Ki-67 within sinusoidal Kupffer cells (Fig. 4-18), there was a significant increase in MT expression in the Ki-67 positive group (median MT expression of 74.6 versus 15.2 cells/25 μm^2, n = 77, P = 0.0045). Median MT expression was also higher in cases containing lymphocytic infiltrates compared to cases with no lymphocytic infiltrate (76.1 versus 25.75 cells/25 μm^2, P = 0.0017; Fig. 4-19). Strong Ki-67 expression was found in all lymphocytic inflammatory foci.

No significant differences were observed in MT expression between positive and negative Ki-67 hepatocyte groups (Fig. 4-20), and no association was found between MT expression and inflammation (Fig. 4-21), fibrosis (Fig. 4-22) and bile duct proliferation (Fig. 4-23). See also Table 4-2.

6.4.1 Results tables

Table 6-1: Inter-observer agreement for two pathologists' histologic scores of all 77 diseased liver samples for hepatic inflammation, fibrosis and bile duct proliferation using the kappa statistic. The kappa statistic is calculated from the observed and expected agreement, which is derived from the distribution of scores for each pathologist. A kappa of 1 indicates perfect agreement, whereas a kappa of zero indicates agreement equivalent to that obtained by chance. A kappa score of 0.41 to 0.60 is interpreted as "moderate" agreement.[167] The *P*–value represents the probability that the calculation of the kappa value is due to chance.

	Observed agreement	**Expected agreement**	**Kappa value**	***P*-value**	**Interpretation**
Inflammation	66.23%	28.25%	0.529	< 0.0001	moderate
Fibrosis	61.04%	32.92%	0.419	< 0.0001	moderate
Bile Ducts	80.52%	54.01%	0.577	< 0.0001	moderate

Table 6-2: Summary table of median metallothionein expression (and interquartile range) for all 77 diseased equine livers for each categorized outcome variable and associated *P*-value (for Dunn's test with post-hoc Sidak adjustment). The median metallothionein expression is measured as the number of positive cells per 25 μm^2. The interquartile range represents the middle 50% of values when ordered from lowest to highest. The *P*-value represents the probability that there is no difference in metallothionein expression by grade for inflammation, and fibrosis, as assessed by pathologist A and B. Total number of observations (n-value) per grade for each category is given.

	Metallothionein expression (number of positive cells per 25 μm^2) per diseased liver and (interquartile range)				
Categorized Variables	**Grade**				***P*-value**
	0	**1**	**2**	**3**	
inflammation (pathologist A)	59 (22.6 - 112.1) n = 8	29.8 (2.4 - 77.6) n = 39	60.3 (15.0 - 97.3) n = 16	74.6 (41.6 - 93) n = 14	0.1423
inflammation (pathologist B)	47.6 (10.3 - 82.6) n = 20	29.4 (0.9 - 83.5) n = 27	74.7 (15.2 - 99.3) n = 21	74.5 (41.6 - 85.1) n = 9	0.0956
fibrosis (pathologist A)	51.2 (44 - 58.4) n = 5	20.35 (6 - 99.1) n = 8	38.7 (4.4 - 99.3) n = 31	49.6 (13.6 - 89.2) n = 33	0.9822
fibrosis (pathologist B)	51.2 (30.4 - 66.8) n = 7	26.4 (3.2 - 89.2) n = 13	35.85 (11 - 87.6) n = 34	74.7 (4.6 - 95.2) n = 23	0.8166

Table 6-3: Summary table of median metallothionein expression (and interquartile range) for all 77 diseased equine livers for each dichotomized outcome variable and associated *P*-value (for Mann-Whitney U-test). The median metallothionein expression is measured as the number of positive cells per 25 μm^2. The interquartile range represents the middle 50% of metallothionein expression when ordered from lowest to highest. The *P*-value represents the probability that there is no difference in metallothionein expression by the presence or absence of bile duct proliferation (as assessed by pathologist A and pathologist B) as well as the presence or absence of Ki-67 expression within bile duct epithelium, lymphocytes, Kupffer cells, and hepatocytes (as assessed by a single observer). The total number of observations (n-value) is provided for each category.

	Metallothionein expression (number positive cells/ 25 μm^2) per diseased liver and (interquartile range)		
Dichotomized Variables	**Absent**	**Present**	***P*-value**
bile duct proliferation (pathologist A)	33 (4.4 - 83.5) n = 51	72.7 (11 - 93.3) n = 26	0.105
bile duct proliferation (pathologist B)	31.7 (4.5 - 81.7) n = 48	74.5 (14.8 - 95.2) n = 29	0.0895
Ki-67 within bile duct epithelium	33 (4.6 - 79.9) n = 67	106.8 (95.2 - 117.5) n = 10	0.0004
Ki-67 within lymphocytes	25.8 (2.4 - 66.8) n = 38	76.1 (27.6 - 99.7) n = 39	0.0017
Ki-67 within Kupffer cells	15.2 (3.2 - 66.8) n = 35	74.6 (30.4 - 95.2) n = 42	0.0045
Ki-67 within hepatocytes	40.7 (9.6 - 87.6) n = 69	74.35 (47.5 - 104.5) n = 8	0.1872

6.4.2 Results figures

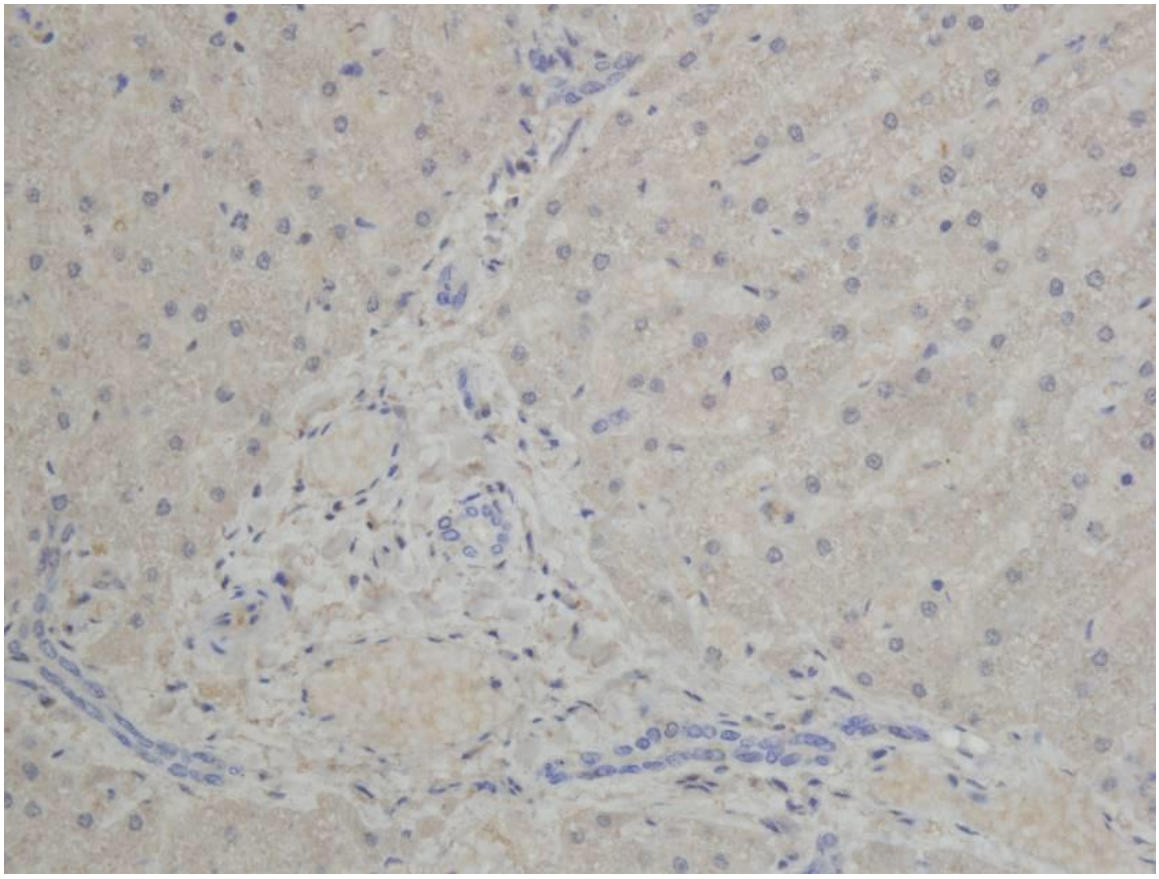

Figure 6-10: A representative image of immunohistochemistry for metallothionein within a histologically normal equine liver. Metallothionein immunoreactivity is absent to low within hepatocytes and staining is uniform (400x).

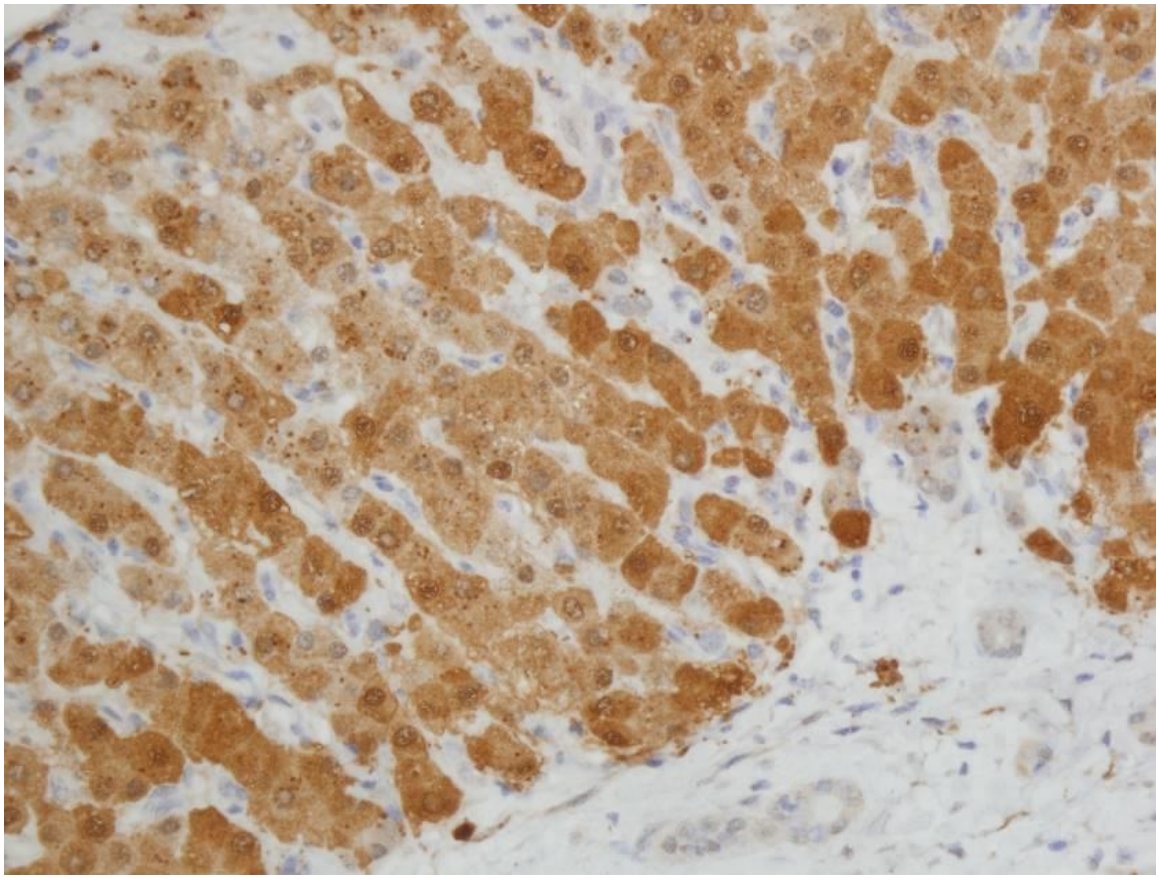

Figure 6-11: A representative image of high metallothionein immunoreactivity within hepatocytes in a section of diseased equine liver. Both cytoplasmic and nuclear staining is present (400x).

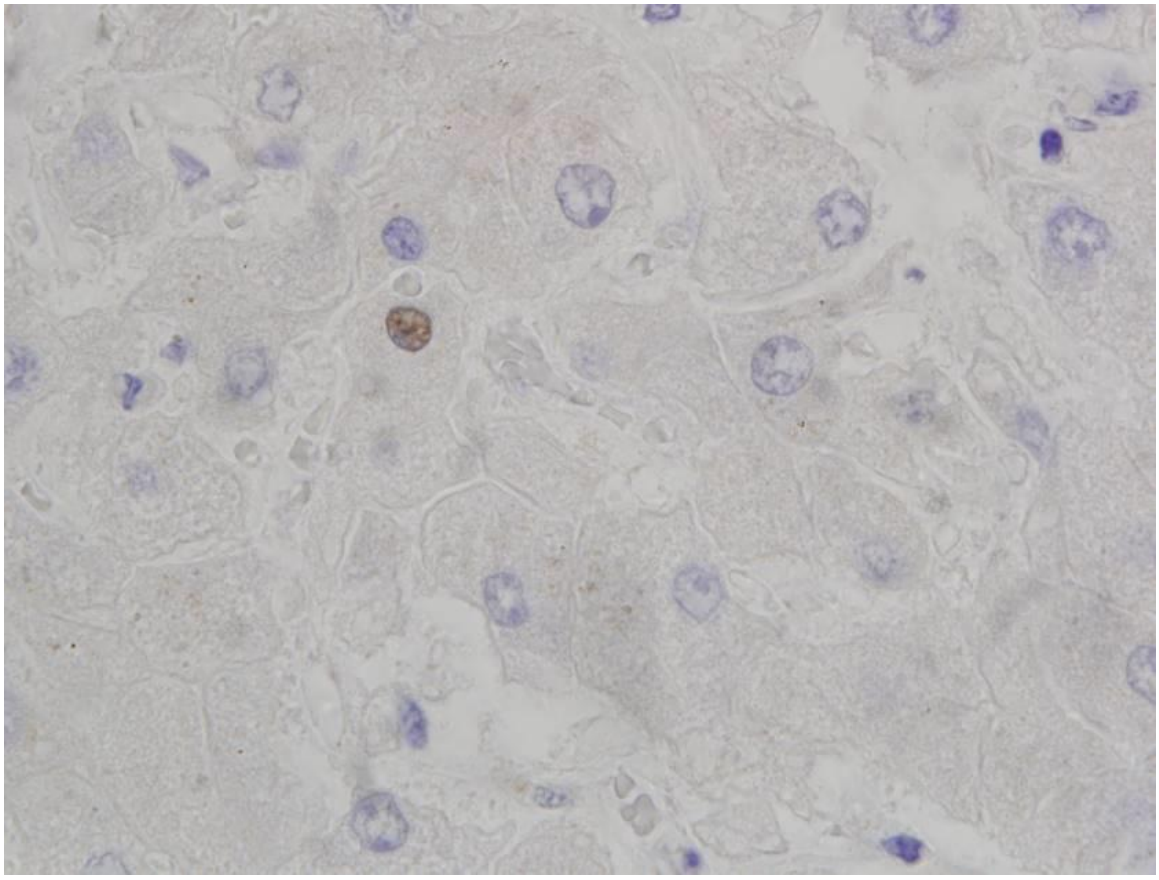

Figure 6-12: A representative image of a single hepatocyte with positive immunoreactivity for Ki-67 within its nucleus in a section of diseased equine liver. 1000x magnification.

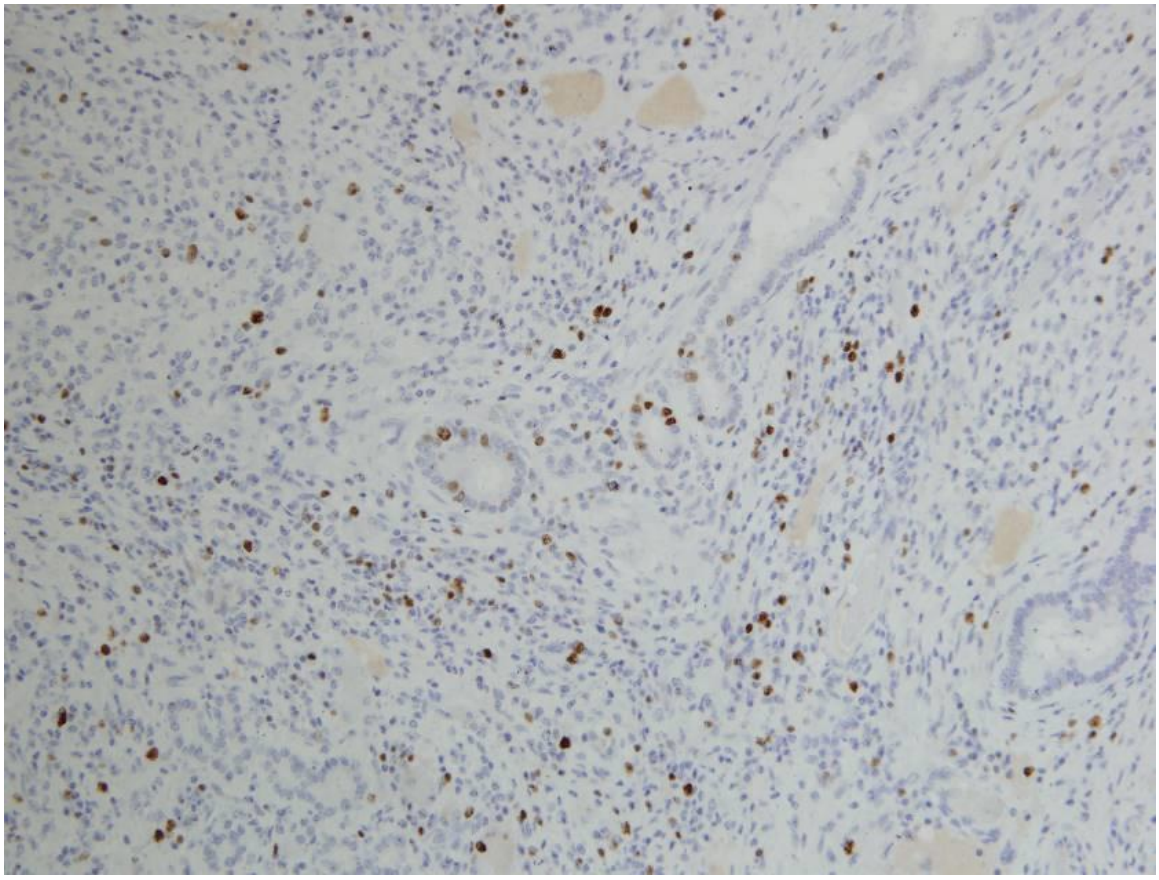

Figure 6-13: A representative image of immunoreactivity for Ki-67 within the nuclei of bile duct epithelial cells and surrounding lymphocytes in diseased equine liver. 200x magnification.

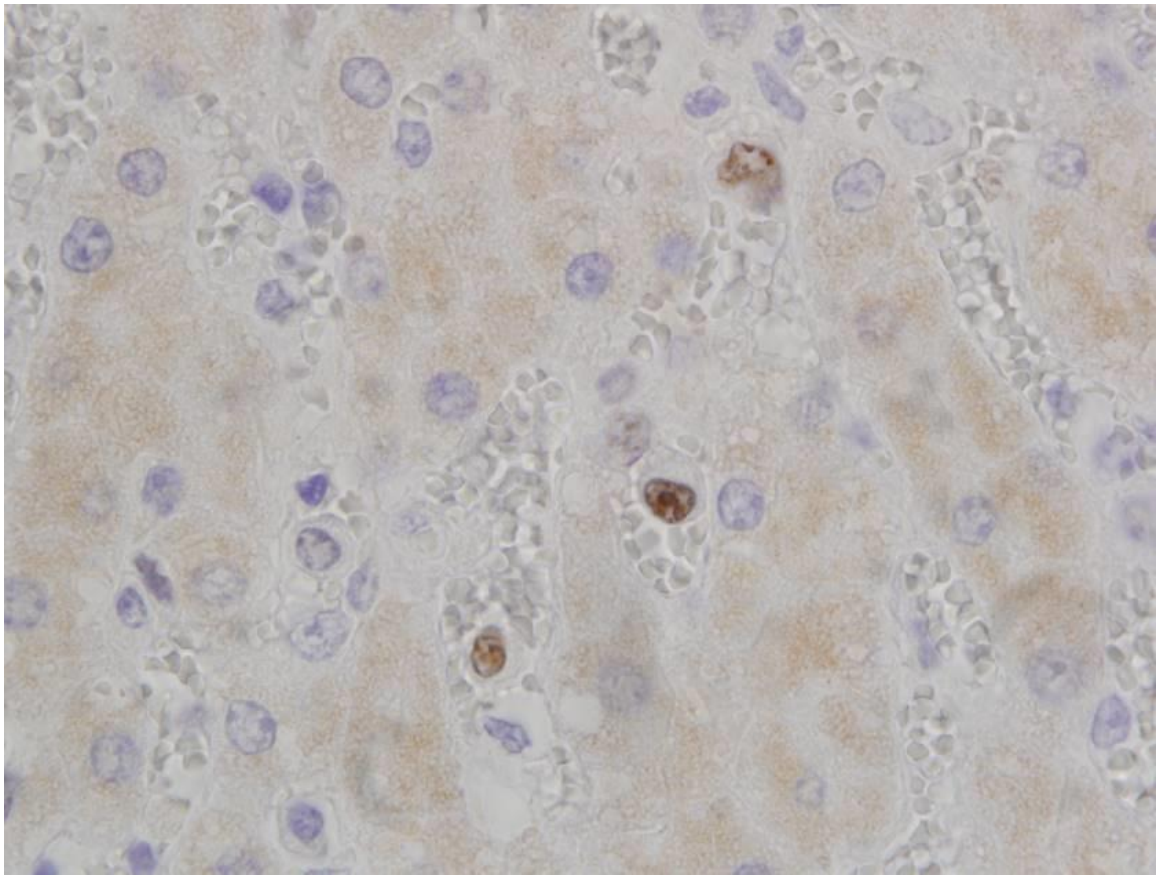

Figure 6-14: A representative image of Ki-67 immunoreactivity within the nuclei of three Kupffer cells (located within sinusoids) in diseased equine liver. 1000x magnification.

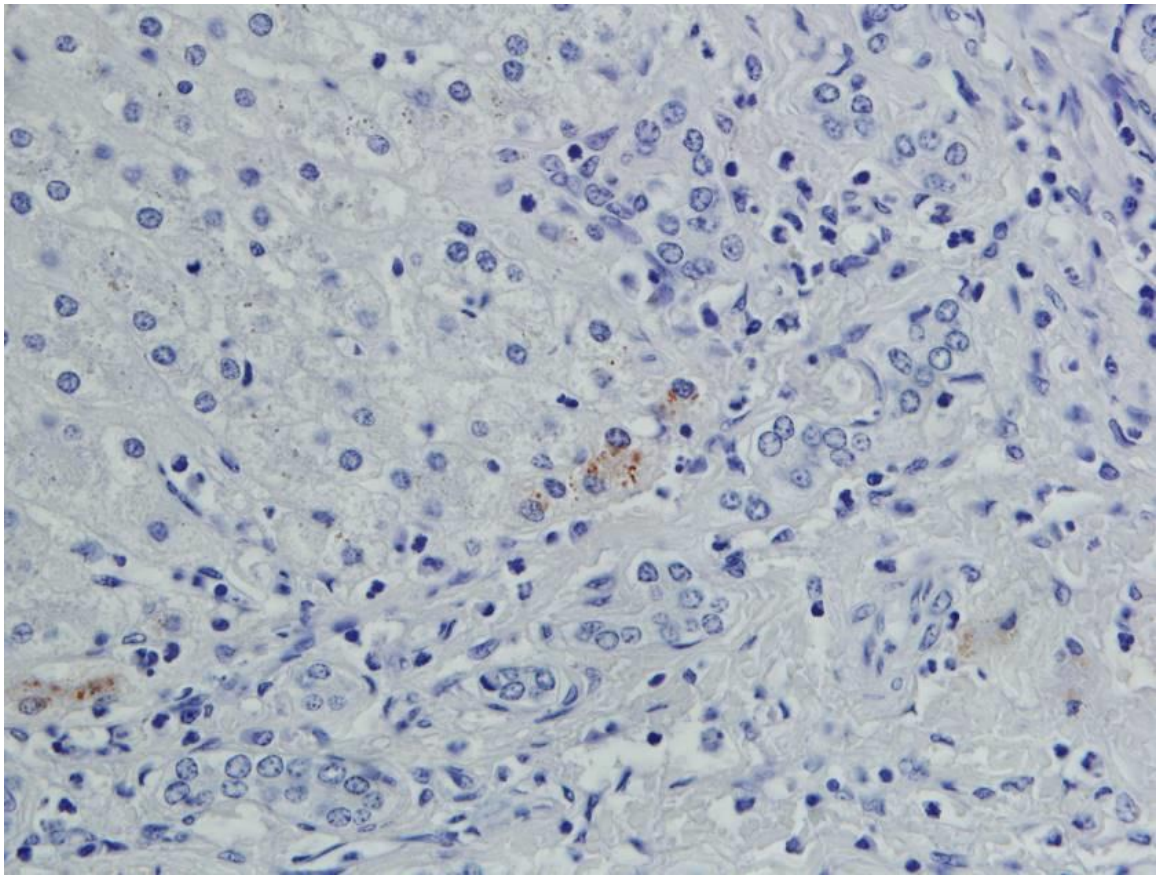

Figure 6-15: A representative image of rhodanine staining (orange-red granules) for copper within the cytoplasm of periportal hepatocytes in diseased equine liver. 400x magnification.

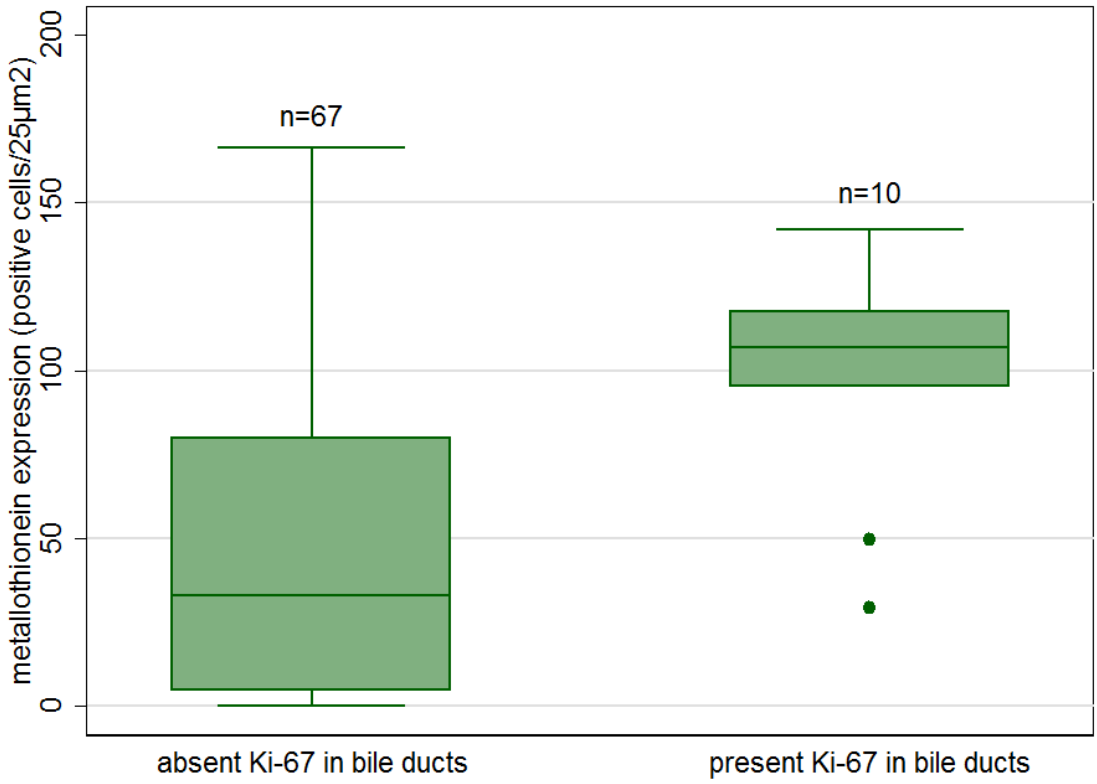

Figure 6-16: Box and whisker plot detailing the distribution of metallothionein expression within hepatocytes by the absence or presence of Ki-67 expression within bile duct epithelium of diseased equine liver. Metallothionein expression was measured as the mean number of positively expressing hepatocytes per 25 µm^2 over 10 fields per liver section. The Mann-Whitney U- test demonstrated a significant difference in metallothionein expression within hepatocytes between diseased livers that demonstrated Ki-67 expression within bile duct epithelium in comparison to those diseased livers that did not (P = 0.0004) A box and whisker plot is interpreted as follows. The green box indicates the 25th and 75th percentile (lower and upper quartile, respectively; also known as the interquartile range), the green line within the box indicates the median of the data distribution. The upper and lower whiskers indicate the most extreme values within 1.5 times the interquartile range of the nearer quartile. The green dots outside the whiskers indicate the presence of outliers which are data values outside 1.5 times the interquartile range. The number of observations in each group is given as an n-value above the whisker.

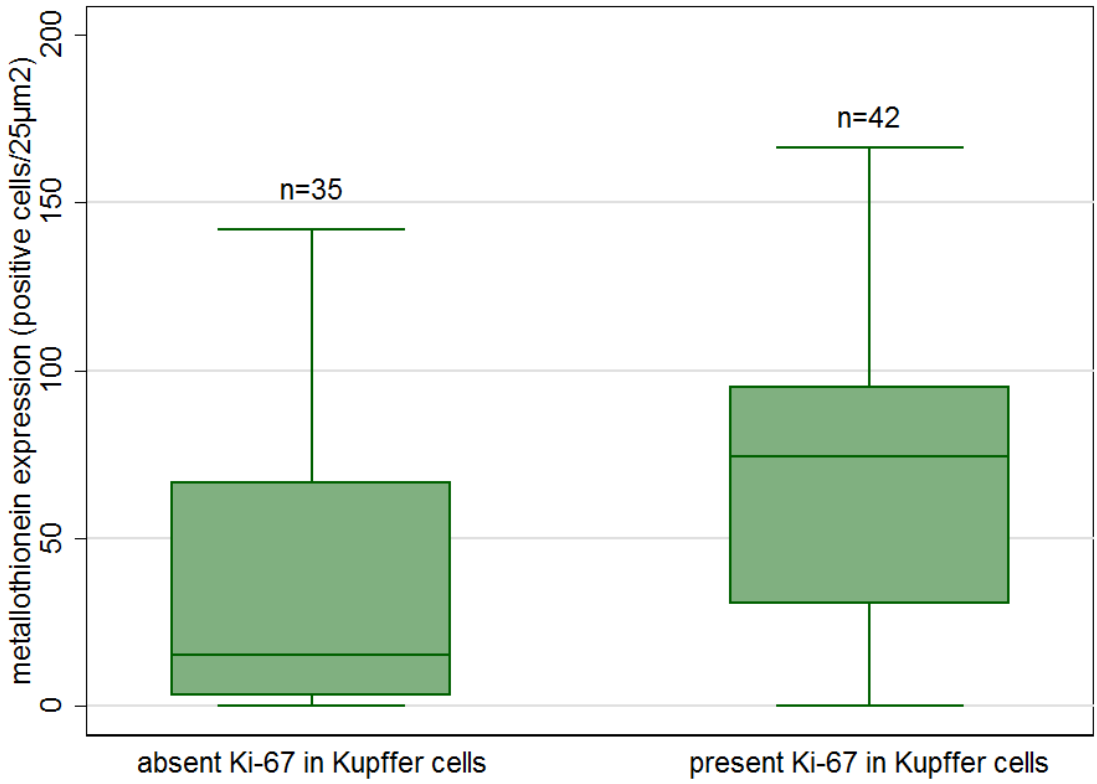

Figure 6-17: Box and whisker plot detailing the distribution of metallothionein expression within hepatocytes by the absence or presence of Ki-67 expression within Kupffer cells of diseased equine liver. Metallothionein expression was measured as the mean number of positively expressing hepatocytes per 25 µm^2 over 10 fields per liver section. The Mann-Whitney U- test demonstrated a significant difference in metallothionein expression within hepatocytes between diseased livers that demonstrated Ki-67 expression within Kupffer cells in comparison to those diseased livers that did not (P = 0.0045). A box and whisker plot is interpreted as follows. The green box indicates the 25th and 75th percentile (lower and upper quartile, respectively; also known as the interquartile range), the green line within the box indicates the median of the data distribution. The upper and lower whiskers indicate the most extreme values within 1.5 times the interquartile range of the nearer quartile. The number of observations in each group is given as an n-value above the whisker.

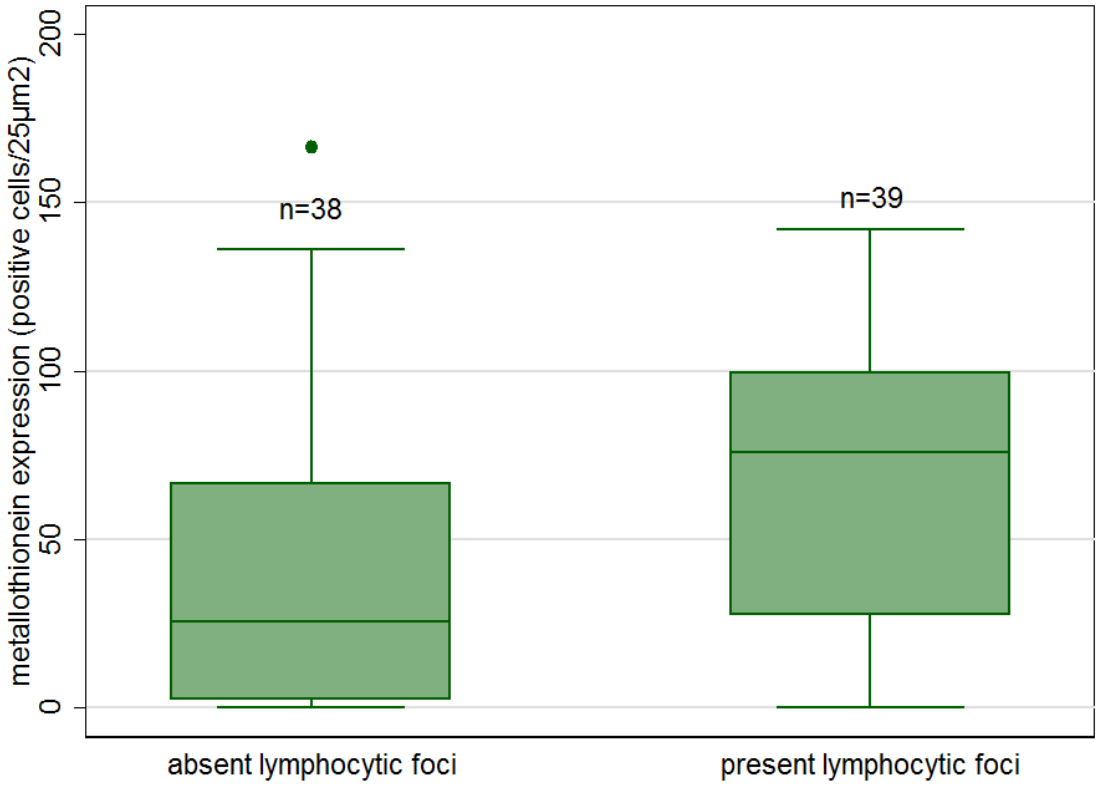

Figure 6-18: Box and whisker plot detailing the distribution of metallothionein expression within hepatocytes by the absence or presence of lymphocytic foci within diseased equine liver. Metallothionein expression was measured as the mean number of positively expressing hepatocytes per 25 μm^2 over 10 fields per liver section. The Mann-Whitney U- test demonstrated a significant difference in metallothionein expression within hepatocytes between diseased livers that demonstrated the presence of lymphocytic foci in comparison to those diseased livers that did not (P = 0.0017) A box and whisker plot is interpreted as follows. The green box indicates the 25^{th} and 75^{th} percentile (lower and upper quartile, respectively; also known as the interquartile range), the green line within the box indicates the median of the data distribution. The upper and lower whiskers indicate the most extreme values within 1.5 times the interquartile range of the nearer quartile. The green dot outside the whiskers indicates the presence of an outlier which is a data value outside 1.5 times the interquartile range. The number of observations in each group is given as an n-value above the whisker.

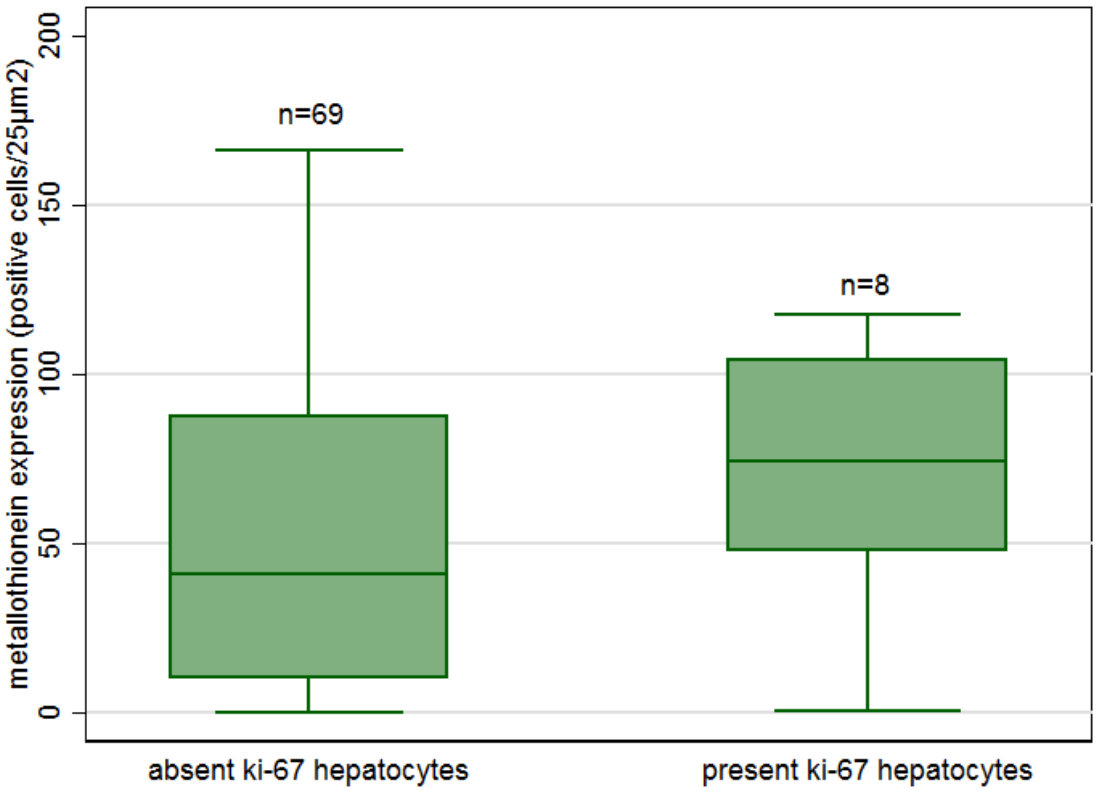

Figure 6-19: Box and whisker plot detailing the distribution of metallothionein expression within hepatocytes by the absence or presence of Ki-67 expression within hepatocytes of diseased equine liver. Metallothionein expression was measured as the mean number of positively expressing hepatocytes per 25 µm^2 over 10 fields per liver section. No significant difference in metallothionein expression within hepatocytes was found between diseased livers that demonstrated Ki-67 expression within hepatocytes compared to those that did not (Mann-Whitney U-test, P = 0.1872). A box and whisker plot is interpreted as follows. The green box indicates the 25th and 75th percentile lower and upper quartile, respectively; also known as the interquartile range), the green line within the box indicates the median of the data distribution. The upper and lower whiskers indicate the most extreme values within 1.5 times the interquartile range of the nearer quartile. The number of observations in each group is given as an n-value above the whisker.

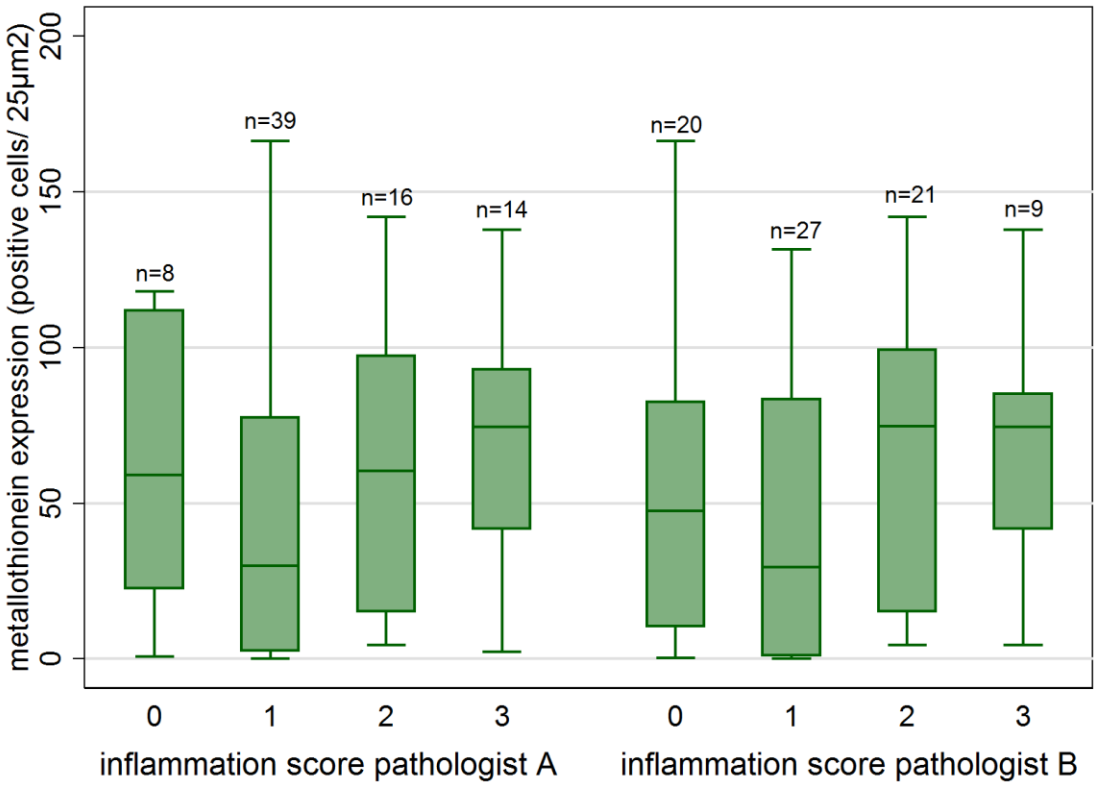

Figure 6-20: Box and whisker plot detailing the distribution of metallothionein expression within hepatocytes by inflammation score as assessed by pathologist A and pathologist B for diseased equine liver. Metallothionein expression was measured as the mean number of positively expressing hepatocytes per 25 μm^2 over 10 fields per liver section. The Dunn's test with a post-hoc Sidak adjustment (to account for multiple comparisons) did not demonstrate any significant differences in metallothionein expression within hepatocytes between groups of different inflammation scores for neither pathologist A or B (P = 0.1423 and P = 0.0955, respectively). A box and whisker plot is interpreted as follows. The green box indicates the 25th and 75th percentile (lower and upper quartile, respectively; also known as the interquartile range), the green line within the box indicates the median of the data distribution. The upper and lower whiskers indicate the most extreme values within 1.5 times the interquartile range of the nearer quartile. The number of observations in each group is given as an n-value above the whisker.

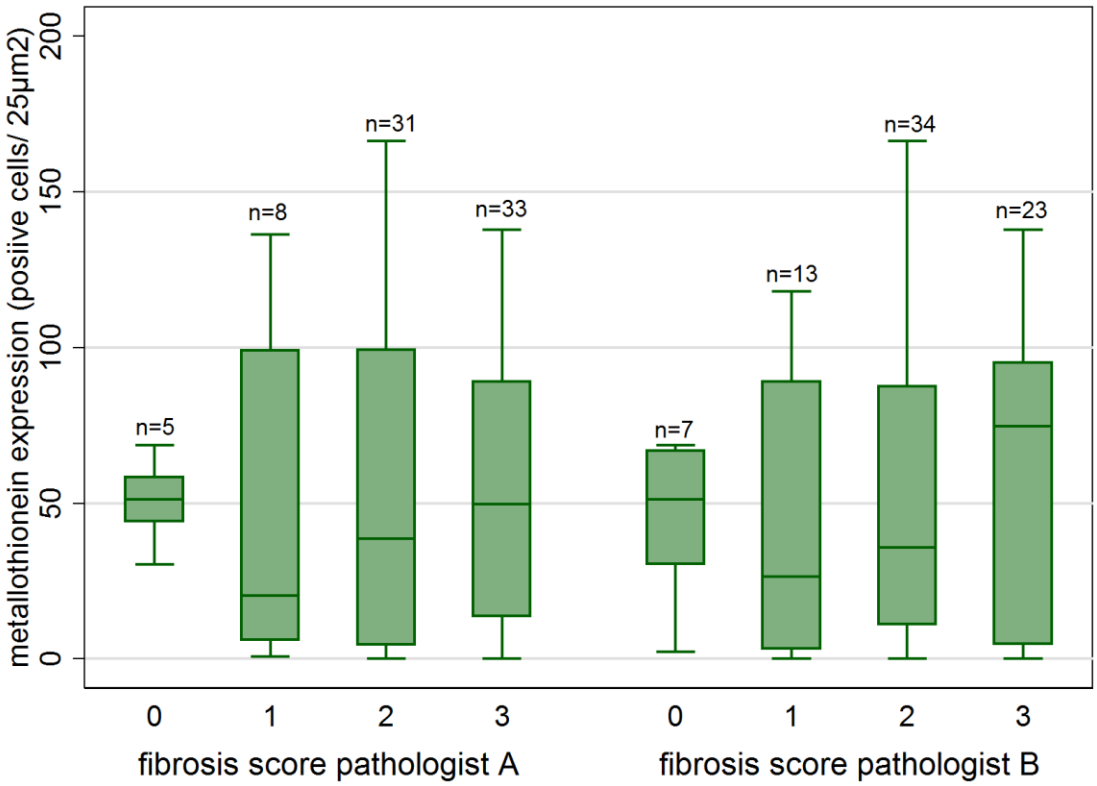

Figure 6-21: Box and whisker plot detailing the distribution of metallothionein expression within hepatocytes by fibrosis score as assessed by pathologist A and pathologist B for diseased equine liver. Metallothionein expression was measured as the mean number of positively expressing hepatocytes per 25 µm^2 over 10 fields per liver section. The Dunn's test with a post-hoc Sidak adjustment (to account for multiple comparisons) did not demonstrate any significant differences in metallothionein expression within hepatocytes between groups of different fibrosis scores for either pathologist A or B (P = 0.9822 and P = 0.8166, respectively). A box and whisker plot is interpreted as follows. The green box indicates the 25th and 75th percentile (lower and upper quartile, respectively; also known as the interquartile range), the green line within the box indicates the median of the data distribution. The upper and lower whiskers indicate the most extreme values within 1.5 times the interquartile range of the nearer quartile. The number of observations in each group is given as an n-value above the whisker.

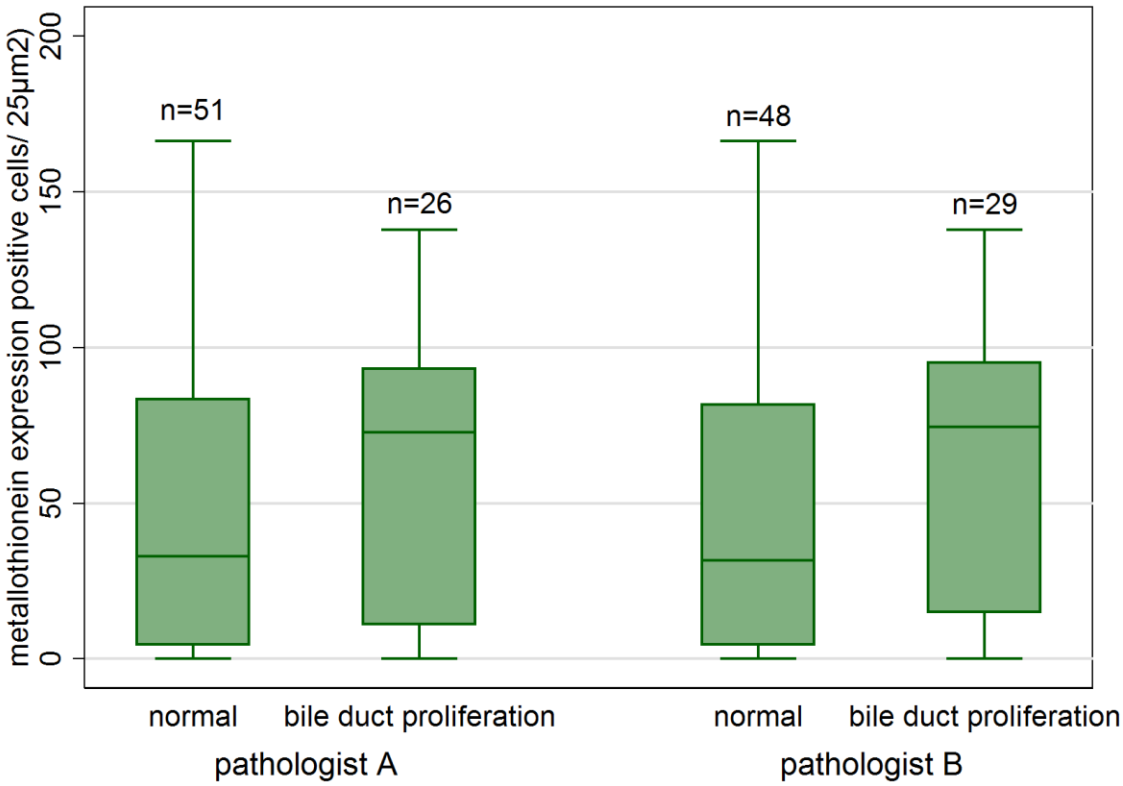

Figure 6-22: Box and whisker plot detailing the distribution of metallothionein expression within hepatocytes by the absence or presence of bile duct proliferation as assessed by pathologist A and pathologist B within diseased equine liver. Metallothionein expression was measured as the mean number of positively expressing hepatocytes per 25 μm^2 over 10 fields per liver section. Cases with greater than four bile ducts per field were considered to have bile duct proliferation, whereas cases with zero to four bile ducts per field were regarded as not proliferated. The cut-off value of four bile ducts per field was determined by utilizing the bile duct counts of six histologically normal liver sections (ten random fields per section) measured by both pathologists. Four or fewer bile ducts per field were found in 95% of the 60 counted fields. No significant difference in metallothionein expression within hepatocytes was found between diseased livers that demonstrated bile duct proliferation compared to those that did not for neither pathologist A nor pathologist B (Mann-Whitney U-test, $P = 0.1050$ and $P = 0.0895$, respectively). A box and whisker plot is interpreted as follows. The green box indicates the 25^{th} and 75^{th} percentile (lower and upper quartile, respectively; also known as the interquartile range), the green line within the box indicates the median of the data distribution. The upper and lower whiskers indicate the most extreme values within 1.5 times the interquartile range of the nearer quartile. The number of observations in each group is given as an n-value above the whisker.

6.5 Discussion

The objective of the current study was to evaluate the role of MT in chronic equine hepatic disease by correlating its expression within hepatocytes with common pathologic lesions seen in the diseased liver, and with cellular proliferation in different cell types within the liver. We report for the first time the presence of a significant relationship between elevated MT expression within hepatocytes and the presence of lymphocytic inflammation or Ki-67 immunostaining within bile duct epithelium and Kupffer cells.

Metallothionein is known to play a role in mediating inflammation,[133] and its gene expression can be induced by cytokines, glucocorticoids, and oxidative stress.[198] Although primarily localized intracellularly within many cell types, MT has also been found extra-cellularly within biological fluids including serum, urine, and milk.[10,193,204,272,280] Interestingly, extracellular MT has been shown to be involved in leukocyte chemotaxis, proliferation, and activation. For example, Yin et al. reported that inflammatory cells migrate towards an increasing MT gradient, a response which can be specifically blocked by antibodies targeting MT.[313] Additionally, MT has been shown to induce the proliferation of B cells and enhance their capacity to differentiate into plasma cells.[27,175] In our study, hepatic MT expression was elevated when Ki-67 was present within lymphocytes. It is, therefore, plausible that MT produced by hepatocytes promotes lymphocyte proliferation in chronic liver disease in horses. Another possibility is that lymphocytic inflammation drives hepatic MT expression.

The significant positive relationship between the presence of Ki-67 immunoreactivity within sinusoidal Kupffer cells and increased MT within hepatocytes further supports the role of MT as an inflammatory mediator in equine liver disease. Intra-sinusoidal Kupffer cells play a major role in the first line of defense against toxic and infectious agents within the liver, both acute and chronic, and activation of these cells causes a release of cytokines, interleukins, and reactive oxygen species.[1,307] Kupffer cells may contribute to hepatocellular injury,[137,168] or may be protective against it,[145] as in the case of acetaminophen toxicity. Additionally, acetaminophen alone had only a minimal effect on Kupffer cells chemotaxis and phagocytosis, suggesting

factors released from hepatocytes themselves may modulate Kupffer cell activation.[168] In a thioacetamide-induced liver injury study by Andres et. al., pre-treatment of rat liver with gadolinium chloride prior to thioacetamide selectively inactivated Kupffer cells, enhanced hepatic MT expression and decreased the overall hepatotoxic effect of thioacetamide in rats.[8] In our study, Ki-67 expression was observed within Kupffer cells, suggesting that these resident macrophages are actively engaged in the cell cycle. It is possible, therefore, that MT produced by hepatocytes modulated the activation of Kupffer cells.

This study also showed that MT might play a role in mitosis of bile duct epithelia in horses, as increased MT expression within hepatocytes was observed when Ki-67 immunostaining was present within bile duct epithelium. Although bile duct proliferation was not significantly associated with MT expression, an increasing trend was observed (Figure 4-23), and non-significance may reflect limited statistical power in this study. MT may play a role in cellular regeneration by regulating the pool of available zinc, which is essential for cell growth and division.[47]

Liver regeneration occurs either by the proliferation of mature hepatocytes, by ductular reaction, or both. Ductular reaction originates from bipotent hepatic progenitor cells (HPCs) within hepatic portal areas and represents an array of reaction patterns depending on the types and severity of hepatic injury.[112] Unexpectedly, very little Ki-67 immunostaining was observed in hepatocytes, despite achieving appropriate levels of immunostaining in each positive control sample. In a previous study, MT was assessed in the diseased canine liver, and Ki-67 expression within hepatocytes ranged from 0.4 to 25.2%.[262] It is plausible that a species-specific pattern of regeneration exists. It is also possible that Ki-67 was absent because these cells reached the resting phase of the cell cycle and were no longer dividing. Alternatively, hepatocyte regeneration may be accomplished less conventionally. For example, in a 30% partial hepatectomy (PHx) mouse model of liver regeneration, regeneration was achieved solely by cell hypertrophy without cell division.[196] In the same study, whereby a 70% PHx occurred, liver regeneration was achieved mainly by cell hypertrophy prior to proliferation. Most cells entered S-phase of the cell cycle, but not all cells progressed to M-phase, whereby liver regeneration was

a result of near equal contributions of hepatocellular hypertrophy and proliferation.[196] However, MT staining was observed within the nuclei of hepatocytes in the current study, suggesting that hepatocytes were regenerating as MT immunostaining within the nucleus is associated with hepatocyte proliferation.[47,280,285] Kupffer cells may also influence differentiation, but not the proliferation, of HPCs during liver injury in mice.[291] Therefore, hepatocyte regeneration in horses may progress by alternative mechanisms, or is significantly slower, and may be achieved by an equal contribution from hepatocytes and bile duct epithelia.

Rhodanine staining for copper was absent in normal liver samples as well as in seven out of ten cases of diseased liver. Only sporadic staining was observed in the remaining three cases. All ten sections of diseased liver submitted for rhodanine staining had elevated levels of MT immunostaining. It is known that MT gene transcription can be induced by the presence of heavy metals such as cadmium, copper, and zinc, [119,155] therefore an investigation into a possible link between copper and elevated MT expression in the equine liver was carried out. Based on the paucity of rhodanine staining, it is likely that copper does not play a role in chronic hepatic disease in horses. Furthermore, there is no obvious connection between copper and MT expression in the equine liver in this study, although this would need to be investigated further with more samples to be definitive. There is only a limited amount of information on hepatic copper levels in horses, though they are relatively resistant to toxicosis.[257] Rare reports do exist of copper causing acute hepatic disease.[12] Additional studies are needed to elucidate the role of copper in the development and progression of liver disease in horses.

Agreement between pathologists was moderate, with the degree of fibrosis being the most difficult lesion to agree upon, despite being provided a rigorous scoring guideline prior to liver section evaluation. Indeed, despite the study inclusion criteria that histologic sections required at least a minimum of hepatic fibrosis, and this was assessed by two pathologists prior to the study, both pathologist A and pathologist B still scored five and seven sections as having 0 fibrosis, respectively. The scoring of histologic sections contains a subjective element and will always have some degree of observer variability. In human medical studies, Cohen's kappa statistic is often used to determine the degree of inter-rater agreement (0=no agreement, 1=perfect

agreement). In general, Kappa values in this study were consistent with other studies involving scoring systems for chronic liver disease.[129]

In conclusion, MT appears to have a role in chronic hepatic lesions in horses, potentially mediated through lymphocytic inflammation and Kupffer cells. Based on these study results, one cannot determine the directionality of this relationship. Metallothionein may potentiate the inflammatory response, or, the inflammatory response may induce or enhance the expression of MT. Furthermore, MT expression may be related to cell proliferation of bile duct epithelial cells, which in turn, may contribute significantly to the regeneration of the equine liver in chronic disease.

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CHAPTER 7:SUMMARY AND GENERAL DISCUSSION

The aim of the studies presented in this thesis was to evaluate the potential role of metallothionein (MT) in chronic liver disease in equids, independent of the cause or etiology. Also, a retrospective study was performed to evaluate the types and frequency of hepatic histopathologic lesions from equids of all life stages, including fetuses, and to compare what is observed in this regional population to what is known in the literature as a whole.

The impetus for this research started with a similar MT project within our research group, in canine chronic hepatic disease.[262] In that study, a statistically significant *positive* correlation between MT expression and cellular regeneration (growth fraction) and a *negative* correlation between hepatic MT expression and fibrosis was observed. In the large body of research involving human hepatic disease, including rodent models, MT has been implicated in numerous inflammatory processes, hepatic fibrosis, as well as cellular regeneration and neoplasia. There is no current literature about the involvement of MT with biliary hyperplasia, though its overexpression has been observed in human cholangiocarcinoma.[245]

The study I presented in this thesis demonstrated that MT, assessed by immunohistochemistry (IHC), plays a role in chronic hepatic disease in equids. MT was shown to be associated with inflammatory processes in the equine liver, potentially mediated through lymphocytic inflammation and Kupffer cells. In addition, it was shown to be associated with biliary epithelial regeneration by Ki-67 expression. As this was the first attempt at assessing hepatic MT by IHC in horses, an equine hepatic MT protocol needed to be developed and validated for this project. When it came time to assess Ki-67 expression, also by IHC, it was very surprising how little was being expressed by hepatocytes, especially when compared to the canine study performed previously. The significance of this finding is unclear, although it is possible that hepatic regenerative mechanisms vary between differing species. The small sample size in each histopathologic score was a limitation to this study and limited the study's power and level of sensitivity.

In conclusion, these findings strongly suggest that MT is involved in chronic hepatic lesions and regeneration in horses. The direction of this relationship during inflammation, however, is still under question. It is known that inflammation can induce the expression of MT, specifically through interleukin-6,[155] and yet it has also been shown that elevated MT levels can change cytokine levels produced by various immune cells.[13] Also, it is unclear if MT works as an anti-inflammatory in this regard, as is most often cited in the literature, or if it acts in a pro-inflammatory manner. This is also the first known account of a possible relationship between MT and the regeneration of biliary epithelial cells. Strong evidence in the literature supports a role for MT in promoting cellular regeneration, especially in the liver. Unlike what was observed in the canine study in which no Ki-67 expression was observed within biliary epithelial cells,[262] Ki-67 expression was observed in equine biliary epithelium, and this was significantly associated with MT expression. This suggests a species-specific mechanism for hepatic regeneration and variability in the role of MT in hepatic regeneration.

The results of the 20-year retrospective analysis are in alignment with what is known from the literature. This suggests that the population in the study shared many similarities with the equine population in general, with respect to hepatic disease, and that the results of the MT study may be applied to the equine population as a whole. Quarter Horses were by far the most represented breed. The most commonly diagnosed hepatic lesions were: suppurative to mixed hepatitis, multi-focal hepatocellular necrosis, and portal fibrosis with bile duct proliferation. All these lesion categories were significantly associated with life stage, suggesting that there is a susceptibility to certain disease entities at distinct stages of development. Not surprisingly, breeds such as donkeys, ponies, and Miniature Horses were significantly associated with hepatocellular vacuolation. What was surprising, however, was the overall increasing trend of suppurative to mixed hepatitis across the study years. Bacterial hepatitis likely is a compelling cause of suppurative hepatitis in equids. However, very few samples overall were submitted for bacterial culture.

Based on these findings, the following recommendations are suggested. Due to the increasing trend of suppurative hepatitis, it is advisable to submit (fresh) samples of liver whenever possible

for bacterial culture. In addition, biopsy is able to detect the majority of lesions in the equine liver. Taken together with any ancillary testing, such as ultrasonography and bloodwork, it can provide valuable information that can guide the practitioner in providing a prognosis or in determining a possible etiology.

As the clinical signs of equine liver disease are vague and only occur once disease has progressed significantly, the development of a rapid and cost-effective screening test for hepatic disease would be incredibly useful to practitioners. Immunohistochemistry is an excellent visualization tool, but quantitative real-time PCR (qRT-PCR) is a much more sensitive detection method. Metallothionein has been accurately detected by qRT-PCR from blood samples determined from dried blood spots on filter paper.[13] If one were able to correlate peripheral blood MT levels with various states of hepatic disease, such as might be accomplished through prospective disease studies, an animal-side or rapid test could be developed. However, caution is warranted as aberrant MT expression has also been observed in other organs and disease processes including Alzheimer's disease,[169] as well as various neoplastic processes.[114]A screening test may also be a useful indicator for performing a liver biopsy.

Another possible application of the results of these studies may involve the development of a histologic scoring system for hepatic biopsies. The scoring system developed in this study could be applied to prospective disease studies and correlated to clinicopathologic findings, survival curves, and etiologies. In this way, biopsy submissions could be evaluated for inflammation, fibrosis, bile duct proliferation in addition to MT expression which may aid in prognosis and therapeutic intervention. For example, it would be interesting to evaluate MT expression with biopsy scores of biliary hyperplasia and fibrosis and clinical outcome. In this way, important prognostic information can be determined in potential cases of chronic toxin exposure, especially since no etiologic agent can be definitively identified.

The role of MT in equine neoplastic processes could also be assessed, and is not limited to hepatic neoplasia, but other organ systems as well. The IHC protocol developed in this study could be applied to other equine tissues.

This is an exciting area of research, with many possible applications to human and animal disease diagnosis and treatment.

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